

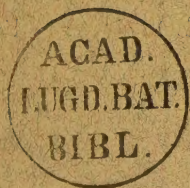
Can Fungi Living in Agricultural Soil Assimilate Free Nitrogen?

A THESIS

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BY

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(WITH EIGHTEEN FIGURES)

I. Historical introduction

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BIBL.

As early as the middle of the nineteenth century it was known to BOUSSINGAULT (cited by SACHS 1) that plants cannot make use of atmospheric nitrogen in their nutrition, but are dependent on combined nitrogen which they ordinarily get from the soil. This idea has become so thoroughly established in all later works that any evidence of nitrogen-fixation by plants is received with unusual interest. Such evidence has come almost entirely from studies of lower organisms, such as bacteria, algae, and fungi.

The full establishment of the nitrogen-fixing power of certain bacteria calls for little discussion in this paper. The important and now classical studies of HELLRIEGEL (2), BEYERINCK (3), HILTNER (4), WINOGRADSKI (5), and STOKLASA (6) have put on a sure scientific basis the fact that a number of bacteria, including not only those associated with root tubercles, but several others, have the power to assimilate free nitrogen, especially when deprived of sufficient quantities in the combined form. It is perhaps worthy of note, also, that this power has been found in many cases to be much augmented by a symbiotic relationship with other organisms, such as algae, Leguminosae, and with other bacteria. A good résumé of the literature of this subject has been given by CHESTER (7), KOCH (8), and HEINZE (9).

With this well established knowledge regarding bacteria, the suggestion has been easy that the closely related organisms, the fungi, might also be found, at least some of them, to possess a similar nitrogen-assimilating power. Acting on this suggestion, a number of investigators within the last 15 years have given attention to this phase of the problem.

Inasmuch as this paper is concerned mainly with this question, a brief historical survey seems desirable. The oldest work on nitrogen-fixation is cited by FRANK (10), and later by FROELICH (11), as having been carried on by JODIN (12) in 1862. A rich fungous growth was observed on nitrogen-free media supplied with sugar, tartaric acid, or glycerin. The purity of the cultures was not certain, although atmospheric nitrogen compounds were excluded and analytical methods were used. Later, BERTHELOT (13) in 1893 reported nitrogen-fixation for three fungi: *Aspergillus niger*, *Alternaria tenuis*, and a species of *Gymnoascus*, cultivated on a medium containing COHN'S (cited by SMITH 14) solution, to which had been added tartaric acid or sugar and kaolin. He claims purity of culture only for *Alternaria*, in which case he reports a nitrogen gain as high as 98 per cent of the original nitrogen content. During the same year, FRANK (15) also reported nitrogen-fixation in certain fungi which he found growing on a nitrogen-free solution of sugar and mineral constituents. Later, using *Penicillium cladosporioides* Fres. (*Hormodendron cladosporioides* Sacc.) on a similar solution, and applying chemical analysis to determine nitrogen gain, he found a gain of 3.5 mg. in a 65 cc. culture.

Further positive results have more recently been reported by PURIEWITSCH (16), SAIDA (17), TERNETZ (18), FROELICH (11), and LATHAM (20). PURIEWITSCH, using *Aspergillus niger* and *Penicillium glaucum*, cultivated on nitrogen-free media with tartaric acid and varying quantities of cane sugar, secured a nitrogen gain of 1.5-8.4 mg. for *Aspergillus* and 2.0-5.2 mg. for *Penicillium*. SAIDA worked with a large number of species, including *Phoma betae*, *Mucor stolonifera*, *Aspergillus niger*, *Endococcus purpurascens*, *Acrostalagmus cinnabarinus*, *Monilia variabilis*, and *Fusicladium moschatum*. He cultivated these on synthetic media with varying quantities of sugar. He also used cultures with and without fixed

nitrogen. In the first four species, he found a nitrogen gain, running as high in one case as 10.53 mg. in a 50 cc. culture of beet sugar decoction. The last three species showed negative results throughout.

TERNETZ first isolated a pycnidium fungus from the roots of *Oxycoccus*, reporting that it had the power of fixing free nitrogen to the amount of 6-10 mg. per 1 gm. of dextrose used. Later, the same author (19) contributed another paper on other pycnidia fungi, isolated from the roots of Ericaceae and referred to the genus *Phoma*. These were cultivated on nitrogen-free media based on WINOGRADSKY'S formula, but using varying quantities of sugar, phosphate, and magnesium. In a full and painstaking investigation, the author reports free nitrogen assimilated by the 5 species of *Phoma* as follows: 2.17, 3.99, 10.9, 18.0, 22.1 mg. per 1 gm. of dextrose used. In the same investigation, *Aspergillus niger* and *Penicillium glaucum* were reported to gain 1.71 and 3.8 mg. respectively per 1 gm. of dextrose used.

Four years later, FROELICH contributed a careful study of four fungi isolated from decaying stems and leaves. The forms were identified as *Alternaria tenuis* Nees, *Macrosporium commune* Rbh., *Hormodendron cladosporium* Sacc., and *Cladosporium herbarium* Link. These were cultivated on a medium similar to WINOGRADSKY'S for *Clostridium pasteurianum*, with quantities of dextrose varying from 2 to 5 gm. per 100 cc., and the cultures were aerated by air freed from combined nitrogen. The author reports nitrogen gains of 2.56-8.92 per 1 gm. of dextrose used. FROELICH also reported confirmation of TERNETZ'S results on *Aspergillus niger* and *Penicillium glaucum*, although much smaller gains are reported by FROELICH.

Perhaps the latest positive results for nitrogen-fixation by fungi have been contributed by LATHAM (20), who worked on *Sterigmacystis nigra* (*Aspergillus niger*), cultivated in solutions containing ammonium nitrate, potassium dihydrogen phosphate, magnesium sulphate, sugar, and a trace of iron. Some of the cultures were modified by the addition of zinc sulphate, but this was reported to have an inhibiting effect on the nitrogen-assimilating power. The cultures without zinc sulphate are reported to fix nitrogen

in quantities all the way from 1.6 to 205.1 mg. The author believes that the critical point of the fungus with regard to the nitrogen supply is slightly below 160.3 mg. of nitrogen in 50 cc. of the solution, and when a greater quantity of nitrogen is supplied to the fungus, it ceases to fix nitrogen and begins to consume it.

All this weight of evidence would seem to establish fully the fact of nitrogen-fixation by fungi, were there not also a large and increasing amount of negative evidence by well recognized investigators. WINOGRADSKY (21) has reported that he failed to confirm the results of BERTHELOT on *Aspergillus niger*, while KOCH (8) states that he was unable to confirm the work of either PURIEWITSCH or SAIDA, giving as his opinion that they worked with impure cultures or were in some other way mistaken in their results. Again, CZAPEK (22) reports negative results for *Aspergillus niger*. Also HEINZE (9) has recorded a repetition of the work of PURIEWITSCH and SAIDA, using similar solutions as well as numerous modifications, including variation of the sugar content and also of the fixed nitrogen content. In no case was he able to confirm the earlier work. He fails, however, to give his analytical data. Further, it may be noted that TERNETZ disagrees quite widely with PURIEWITSCH regarding the amount of nitrogen gain in the case of *Penicillium* and *Aspergillus* in nitrogen-free solutions. It may be added further that BREFELD (23) and HEINZE (9) have reported negative results with reference to the nitrogen-fixing power of certain species of *Ustilago* and with *Dematium*-like fungi and yeasts.

More recently, further weight has been added to the negative side by the work of PENNINGTON and DUGGAR, as well as by the work of this investigation. PENNINGTON (24) first reported no nitrogen-assimilating power for a species of *Fusarium* isolated from the soil. Later (25) he reports absolutely no nitrogen-fixing power for *Aspergillus niger*, *Penicillium glaucum*, and *Alternaria* (sp.), either in nitrogen-free or in nitrogen-containing media, using methods similar to those of FROELICH and TERNETZ. DUGGAR (26) has very recently reported negative results for the following fungi: *Coprinus comatus*, *Daedalea quercina*, *Polyporus sulphureus*, *Trichoderma lignicola*, and *Aspergillus niger*. With these he carried

out two series of experiments, involving about 400 flask cultures with a great variety of culture media. Analyses showed that in no case was there fixation of atmospheric nitrogen, except possibly in certain cultures of *Aspergillus niger*. In many cases, DUGGAR reports there was a loss of nitrogen, which he attributes, usually at least, to the production of gaseous nitrogen. A third series of experiments is referred to, in which additional fungi have been tested and the experiments of other investigators have been duplicated with negative results.

It is beyond the province of this paper to consider the mycorrhiza fungi, any more than merely to refer to the work of HILTNER (27), FRANK (28), and others, who have reported nitrogen-fixation by such fungi.

Discussion of these apparently very conflicting results will be reserved till a later part of this paper, the purpose here being only to give a brief historical review of the work on this problem up to the present time.

The investigation described in this paper was begun several years ago, under the direction of Professor J. B. POLLOCK at the University of Michigan. The following purposes were in mind: (1) to determine what species of fungi live habitually in an ordinary agricultural soil; (2) to study their distribution as to depth and nature of the soil; and (3) to begin some study of the part they play in soil fertility. After isolating and making a study of about 17 species, attention was turned to the problem of nitrogen-fixation, since it was thought, from our knowledge of bacteria, that if any fungi possessed nitrogen-fixing power, such forms might well be looked for in the soil. Furthermore, if any soil fungi were found to fix nitrogen to any marked degree, such a fact would have important bearing on soil fertility, as well as a large interest from the purely physiological standpoint.

II. Isolation and identification of soil fungi

HISTORY OF SOIL FUNGI INVESTIGATIONS

Although many of the fungi living in the soil have been known for some time, and many attempts have been made to isolate mycorrhiza forms, little work seems to have been done previously

in studying the fungous flora of the soil as such. This is the more surprising, since what study has been carried on indicates that there is a very abundant and distinct group of forms which live habitually in the soil.

Perhaps the most extensive work in isolating and studying these forms is that of OUDEMANS and KONING (29), who reported 45 species from a humous soil of the forest of Spanderswoud, near Bussum, in Holland. It seems quite remarkable that of these 45 species, 32 were named as new species which inhabit the soil. These belonged to the Hyphomycetes and Phycomycetes. Few other similar studies seem available, although ADAMETZ (30) has carried on a study of organisms found in a field soil. He reports having isolated, besides a number of bacteria and yeasts, the following fungi: *Penicillium glaucum*, *Mucor mucedo*, *M. racemosus*, *M. stolonifer*, *Aspergillus glaucus*, and *Oidium lactis*. Again, HAGEM (31) has recently reported an investigation of Mucorineae which he isolated from soil and air; 16 species were obtained from the soil, and it is interesting to note that he named 8 of these as new species.

Since the completion of this investigation, and after the appearance of a preliminary paper by the author (Proc. Mich. Acad. Sci. 1911), a paper by DALE (46) on soil fungi has been received. This investigator reports and figures 20 genera of fungi isolated from a sandy soil obtained from the Royal Agricultural Society's Farm at Woburn, England. The resemblance between DALE's list and that of this paper is certainly very striking. Not only many of the genera, but a number of the species, are the same. I note also in DALE's paper a reference to a recent paper by JENSEN (47). It is evident from these numerous recent studies that much attention is being given to this subject.

SELECTION OF A SOIL PLAT

For the isolations of this investigation, a plat of rather rich, clay loam was selected from a garden in Ann Arbor. It had been in use for many years for raising common garden vegetables, usually in rotation. As a rule, it had been heavily manured, but during the last few seasons this had been less frequent and less

abundant. No manure had been used the year previous, although a garden crop had been raised. The entire plat was 20×60 feet. This was divided into three equal squares which were treated as follows: plat I was untilled and unfertilized; plat II was well tilled but unfertilized; while plat III was both well tilled and well fertilized with stable manure. Samples of the soil from these plats were taken in a manner hereafter described, and cultures were made in the laboratory by the usual method, outlined by DUGGAR (32, pp. 34-40). Pure cultures were then isolated and studied.

CULTURE MEDIA

Media for the platings and pure cultures were chosen with reference (1) to the idea of inhibiting the growth of bacteria, and (2) to getting a firm medium on which fungi could be studied conveniently. Two methods were tried: (1) a large percentage of gelatin was added to the medium, and (2) the medium was made strongly acid by the use of oxalic or lactic acid. The latter method was abandoned, proving largely a failure on account of the tendency toward liquefaction following sterilization. The first method was successful, and was therefore employed for the first platings throughout the investigation. The only difficulty in its use was a tendency with the mineral salts used to give a precipitate, probably magnesium sulphate, during the sterilization. Less of this difficulty occurred by the use of the steam sterilizer than with the autoclave. By the former method a fairly clear medium was obtained, on which practically no bacterial growth appeared.

For the pure culture tubes, an agar medium without gelatin was found most satisfactory, on account of the easy liquefaction of the gelatin in warm weather. Again, a medium containing calcium nitrate instead of ammonium nitrate was found to produce more characteristic cultures of some fungi, since with the latter a cheesy consistency was developed on the medium, and a slimy crust was often formed over the surface, which interfered with the vegetative development. After considerable experimentation, three media were finally used: medium 1 was used almost exclusively for the first platings; the growth of the fungi was also tested on this medium in pure cultures; media 2 and 3 were used ex-

clusively for the pure cultures. These media had the following composition:

MEDIUM 1

Distilled water	100.0 cc.
Gelatin	30.0 gm.
Agar	2.0 gm.
Monopotassium phosphate	0.2 gm.
Ammonium nitrate	0.2 gm.
Magnesium sulphate	0.02 gm.
Sodium chloride	Trace

MEDIUM 2

The same as medium 1, except the gelatin was omitted.

MEDIUM 3

Water	100.00 cc.
Agar	2.00 gm.
Monopotassium phosphate	(M/100) 0.136 gm.
Calcium nitrate	(M/100) 0.164 gm.
Magnesium sulphate	(M/1000) 0.012 gm.
Cane sugar	(M/100) 0.270 gm.

Medium 3 was not quite so transparent as medium 2. However, as previously noted, the fungi studied seemed to develop more characteristically on the latter. The acidity of media 1 and 2 was tested by the method in common use among bacteriologists (14), described by DUGGAR (32). Medium 1 gave an acidity of 80, according to FULLER'S (34) scale; medium 2 gave 20. The inhibiting effect of the gelatin medium on bacteria is thought to be due to its rather strong acidity. The quantities of the mineral constituents of these media were based on some studies on "optimum media" carried on in this laboratory, where it has been found that the optimum quantities, especially of the nitrate, are much below the quantities generally recommended. The same has been confirmed in this investigation.

METHOD OF SAMPLES, PLATINGS, AND ISOLATIONS

The method of taking the soil samples was a slight modification of that used by KING and DORYLAND (35), at the Kansas Experiment Station, in bacteriological work. The sampler consists of a steel tube, 10 cm. long and 1 cm. inside diameter, together with a

brass rod, 11 cm. long and 1 cm. in diameter, made to operate as a piston inside the tube. At one end of the tube is a collar through which operates a small thumbscrew for holding the piston in any position desired. The opposite end is sharpened for entering the soil. The brass rod is graduated so as to indicate the size of each soil sample in cc. At the thumbscrew end, the brass rod is rounded to form a convenient knob for holding. For use with the sampler, a lath 20 cm. long and 5 cm. wide was perforated with a row of holes, each slightly larger than the sampler, running lengthwise of the lath and 2 cm. apart from center to center.

The samples were taken as follows: first a hole, about 30 cm. square and 20 cm. deep, was dug with a spade in the experimental plat. Then a vertical face of this hole was scraped off with a sterilized steel spatula, and the fresh surface immediately covered by the lath. Next, the sampler, having been set for the size of sample desired, and sterilized in an alcohol flame immediately before use, was pushed horizontally into the soil through one of the holes in the lath at the desired depth. After being turned once or twice on its long axis to loosen the sample, the sampler was removed and the sample was pushed quickly into a sterilized, cotton-stoppered test tube provided for the purpose. The cotton plug was held between the fingers while putting in the sample, and was returned as quickly as possible. By this method, all surface contamination was avoided and only fungi actually existing in the soil at the depth of the sample were taken. A view of the whole sampling outfit is given in fig. 1.

Samples of 2 cc. each were taken, and after being transferred to the sterile, cotton-stoppered test tubes, were carried to the laboratory and each treated with 18 cc. of sterile distilled water. Each sample was thoroughly shaken, and as soon as the coarser soil particles had settled, 2 or 3 drops were transferred, by means of sterilized pipettes, to the first of a series of three tubes, each of which contained 10 cc. of the gelatin medium, which had been melted previously and maintained at 42°-46° C. From the first of these three tubes 2 or 3 drops were transferred to the second, and from the second 2 or 3 drops to the third. Plates were then poured in the usual way.

Vigorous growth was generally obtained in 3 or 4 days, this time being shortened by higher temperatures. As fast as individual mycelia could be distinguished, pure cultures were isolated by transferring to properly sterilized tubes of media. Medium 2 or 3 was usually used for this purpose, on account of the tendency of the gelatin to liquefy in warm weather. Little difficulty was encountered in getting pure cultures in this way. That the method of plating was successful in keeping out all foreign spores was demonstrated by a set of blanks, which was made at the time the other plates were poured. These were poured like the others, but

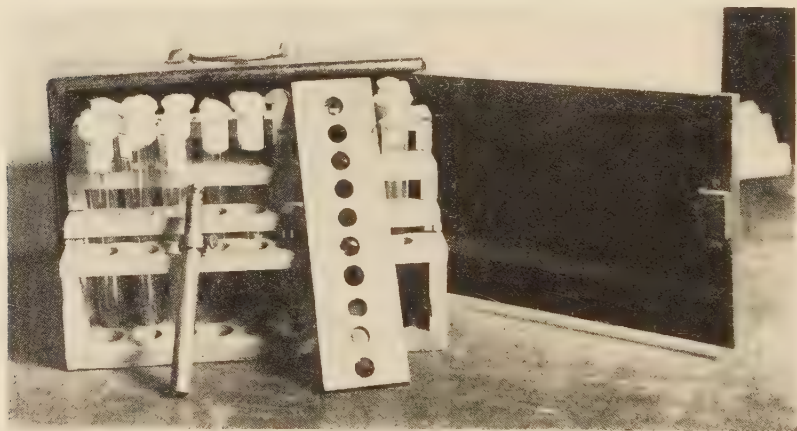


FIG. 1.—Sampling outfit

were inoculated from sterile distilled water. On these, no fungi developed until many days after liberal growth had occurred on the soil-inoculated plates, and then only an occasional mycelium appeared on the very edge of the plate. In most cases, these plates were perfectly clear for weeks after their preparation.

DISTRIBUTION AS TO DEPTH

In accordance with a previously stated purpose, these fungi, after isolation, were studied with reference to the following points: (1) distribution as to depth; (2) distribution as to treatment of soil; (3) structural characters and identification. These will be considered in the order given.

The studies of distribution according to depth were made on all three of the plats previously indicated. Samples were taken in different positions horizontally on each plat. Three depths were tried at first: 2 cm., 8 cm., and 14 cm. Later, samples were taken at only two depths: 4 cm. and 12 cm. Four sets of samples were taken on dates and at depths indicated in table I. A series indicates a set consisting of a sample at each level given.

TABLE I

Date		Plat	No. of samples	Depths in cm.
April	28.....	I	2 series	2, 8, 14
July	12.....	I	2	2, 8, 14
	12.....	II	2	2, 8, 14
August	15.....	III	1	2, 14
October	12.....	I	1	4, 12
	12.....	II	1	4, 12
	12.....	III	1	4, 12

After mycelia had become 3–8 cm. wide, countings were made of each plate, as accurately as possible without special device, to determine the number of mycelia for each plate. The counting for each sample included all the mycelia on the three plates which were inoculated from that sample. A summary of the results obtained by these countings is given in table II. The averages given were obtained by adding together all the countings made at a particular depth and then dividing by the number of samples at that depth.

TABLE II

PLAT	AVERAGE NUMBER OF MYCELIA PER SAMPLE AT EACH DEPTH				
	2 cm.	4 cm.	8 cm.	12 cm.	14 cm.
I.....	68.6	21.5	52.3	29.0	54.3
II.....	101.5	32.5	59.5	35.5	50.0
III.....	17.8	36.5		28.0	20.0
Average for 3 plats.....	62.6	30.2	55.9	30.8	41.4

While the accuracy of the absolute values of these countings, from which these averages are computed, might well be questioned, nevertheless, it is believed that the relative values of the averages

are significant. The method of counting was probably not strictly accurate, and, furthermore, the difference in the times of taking samples might well affect the absolute numbers. It is also true that the investigation of a larger number of samples would be desirable before drawing final conclusions. Nevertheless, the comparative results indicate that the fungi in the soil investigated were distributed rather uniformly at different depths, at least as low as 14 cm. No samples were taken at a greater depth. This result is quite different from that usually found for bacteria (see KING and DORYLAND 35).

Some study was also made of the depths at which particular fungi were found. From the results obtained, no definite relationship was discovered. Any particular species seemed about as abundant at one depth as at another, down as deep as samples were taken. The results for the three most abundant species are given in table III.

TABLE III

NAME	TOTAL NUMBER OF ISOLATIONS	NUMBER OF CULTURES AT EACH DEPTH				
		2 cm.	4 cm.	8 cm.	12 cm.	14 cm.
<i>Fusarium</i> (sp. ?).....	13	2	1	5	3	2
<i>Mucor ambiguus</i>	14	4	3	1	3	3
<i>Trichoderma nigro-virens</i>	8	3	4	0	0	1

These data were obtained, not from the original plate cultures, but only from the pure cultures isolated from the plates. This was for the reason that the fungi were many of them not identified until after they had been isolated for some time, when those in the plates had overgrown each other too far to be distinguishable. The data here given are regarded as insufficient for the drawing of final conclusions, but as far as they go, they do not seem to show any definite relationship between the position of particular species and depth.

DISTRIBUTION ACCORDING TO TREATMENT OF SOIL

As previously stated, the three plats were differently treated as to tillage and fertilization. It was expected that a marked difference would be found in the flora of the different plats, espe-

cially in plat III as compared with the others. This plat was heavily manured, and then well spaded and raked. The results, however, did not seem to fulfil the expectation mentioned. Out of about 60 pure cultures isolated, representing 18 different species, but 2 species were found exclusively on plat III, while 3 were found exclusively on plat I, and 3 on plat II. It would seem more reasonable to suppose that these differences were due rather to the chances of sampling or isolation than to differences in the flora of the plats. The results would indicate then a probability, at least, that there is a rather constant and characteristic fungus flora in the soil, regardless of the treatment as to tillage or manuring. It should be noted in this connection that the samples from the manured plat were not taken until about 3 months after the manure was applied. Any foreign fungi, therefore, which might have been introduced with the manure may have begun to grow, and, finding conditions unfavorable, have died out.

The conclusion that there is a rather constant and distinct fungus flora in the soil is strongly confirmed by the work of other investigators. Mr. GROSSMAN, whose work has not yet been published, has worked in this laboratory on a very different soil from that used in this investigation. The soil used by him was a very fine, red clay on which plants grow poorly, located about a mile away from the University. Out of about 12 or 14 species isolated from this clay soil, at least 8 are the same as those reported here. What seems even more remarkable in this connection is that out of 18 species isolated here, 7 are the same as those found by OUDEMANS and KONING (29) in forest soil near Amsterdam, Holland. And furthermore, 4 of these were named by OUDEMANS as new species. Out of 13 genera found here, 8 were the same as those found in Holland.

LIST OF FUNGI ISOLATED

The list of fungi isolated is as follows (p. 262). One asterisk indicates fungi found also by GROSSMAN; two asterisks, those found also by OUDEMANS and KONING (29); three asterisks, those found in all three investigations. The descriptions follow, and drawings may be found in figs. 2-16.

PHYCOMYCETES.—(1) *Mucor ambiguus* Vuill.; (2) *M. stolonifer* Ehrenb.

HYPHOMYCETES.—(3) * *Myceliophthora sulphurea*, n. sp.; (4) *Coccospora agricola*, n. sp.; (5) * *Fusarium*, sp.; (6) ** *Acrostalagmus cinnabarinus* Cda.; (7) * *Pachybasium hamatum* (Bonord); (8) ** *Aspergillus calyptratus* Oudem.; (9) *A. nidulans* (Eidam); (10) * *Penicillium glaucum* Link; (11) * *P. bicolor* Fries; (12) * *P. candidum* Link; (13) ** *P. humicolum* Oudem.; (14) * *Hormodendron cladosporioides* Sacc.; (15) ** *Monilia Koningi* Oudem.; (16) *** *Stysanus stemonites* (Pers.); (17) *Trichoderma nigro-virens*, n. sp.; (18) ** *T. Koningi* Oudem.; (19) *Verticillium chlamydosporium*, n. sp.

DESCRIPTIONS OF FUNGI

For the identifications the systematic works of ENGLER and PRANTL (36), RABENHORST (37), and SACCARDO (38) were used. The work of OUDEMANS and KONING (29) was also found very useful, and the work of LÈGER (40) was referred to.

MUCOR AMBIGUUS Vuill.—*Mycelium* floccose, spreading rapidly, soon covering the substratum and sending aerial hyphae 1–3 cm. above; becomes dusty gray by formation of sporangia. *Hyphae* branched, hyaline, 6–10 μ in diameter. *Sporangiophores* very variable in length; branching racemose-sympodial, richly mixed. Each method occurs separately and the two are often combined in the same fructification. In the racemose type the terminal sporangia are often larger than the lateral, the whole appearing clustered. In the sympodial branching a cross wall cuts off a sporangiophore, and then a lateral branch forms basal to this wall. The lateral branch continues growth, repeating the process, thus developing a sympodial system. The two types of branching are often combined by one or more branches of a racemose cluster becoming elongated and developing in the sympodial way. Branches of the sympodium 10–100 μ long. *Sporangia* globular, 60–100 μ in diameter, larger terminal sporangia 125 μ , dusty brown in color. *Columella* present, conical to globular, 35–50 $\times$ 40–60 μ in diameter, slowly deliquescent leaving a slight collar. *Spores* elliptical, seldom globular, 3–5 $\times$ 5–7 μ in diameter, smooth.—Fig. 2.

This fungus showed extreme variation in the branching of its sporangiophores. While its diagnosis, so far as branching is concerned, comes very near to *M. globosus* Rabenh., its spores are typically elliptical and not globular. At the same time, the columellas are conical to globular and not pyriform.

These characters correspond much more closely to *M. ambiguus*. Considering all its characters, it was decided to refer it to that species.

This form was found very abundantly in all the cultures made. Its abundance was exceeded only by that of *Fusarium* sp.

Myceliophthora sulphurea, n. sp.—*Mycelium* at first orbicular, white, loose, and tufted in the center with a radiate border; becomes sulphur yellow and finally zoned with pale yellow or white center, alternate white and yellow ridges, and a white, radiate border; reverse side orange yellow; medium little colored. *Hyphae* branched, septate, hyaline, 3–4 μ broad, easily falling apart into oidia-like segments of very variable size and form, some rounding up into oval or globular conidia, others remaining as undifferentiated cells. *Conidiophores* scarcely differentiated from the mycelium; sometimes whole hyphae break up into oidia, in other cases rows of conidia, with yeastlike branching, form at the ends of certain branch hyphae. *Conidia* exceedingly variable, from undifferentiated cells to oval globular spores 5–10 $\times$ 10–16 μ in diameter; no chlamydospores.—Fig. 3.

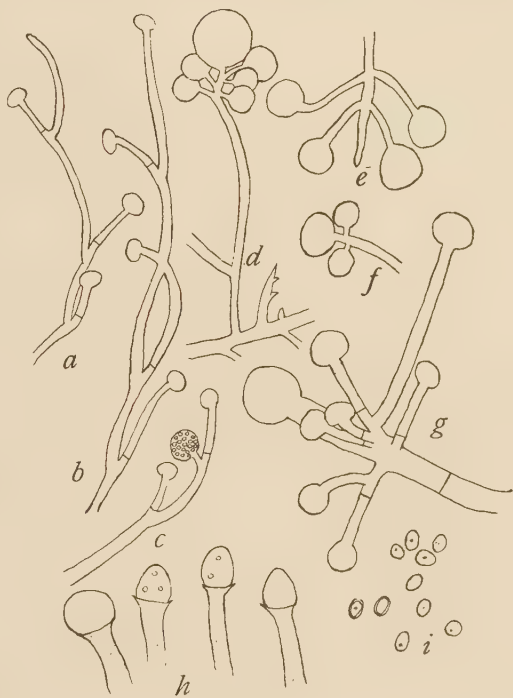


FIG. 2.—*Mucor ambiguus* Vuill.: a-c, sympodially branched sporangiophores, $\times 80$; d-f, racemose sporangiophores, $\times 80$; g, mixed racemose-sympodial sporangiophore, $\times 180$; h, columellas, $\times 180$; i, spores, $\times 380$.

The vegetative appearance of this form is very characteristic and very constant, but the spores and method of fructification are rudimentary and very irregular.

Coccospora agricola, n. sp.—*Mycelium* orbicular, at first white with a tufted center and radiate border, becoming slightly zoned with concentric grooves and ridges, turning pinkish brown especially inside, finally forming a powdery pinkish brown surface with age; reverse side weakly orange. *Hyphae* very little branched, septate, 4–6 μ broad, hyaline. *Conidiophores* little differentiated, consisting



FIG. 3.—*Myceliophthora sulphurea*, n. sp.: a, habit sketch; b, c, e, hyphae bearing simple conidiophores with conidia, $\times 180$; e–h, hyphae breaking up into oidia, $\times 380$; i, oidia-like conidia, $\times 380$.

of short side branches, each bearing a single spore at the end, or sometimes forming racemose clusters; side branches 12–30 μ long, generally septate. *Conidia* (chlamydospores) large, thick-walled, mostly globular, very persistent, not being set free in water, 16–25 μ in diameter; membrane hyaline, 2–3 μ thick; contents highly granular and faintly brownish.—Fig. 4.

The large, thick-walled, persistent chlamydospores, and the simple method of fructification, were the most strikingly characteristic features of this fungus.

FUSARIUM (sp. ?).—

Mycelium white, changing to pink, spreading rapidly to form a compact, floccose mat of indefinite extent, soon covering the medium; no zonation, reverse color pink, medium colored pink. *Hyphae* profusely branched, septate, rising to form a loose, cottony web above the medium, granular, hyaline, 2.5–3.5 μ broad, under unfavorable conditions forming knotted chains of chlamydospores. *Conidiophores* at first short side branches bearing a spore at the end, or lacking with the

spore sessile; later developing into a *Cephalosporium* stage in which the conidiophore lengthens, becoming $30-80\ \mu$ long, cutting off successive conidia (microconidia) at the end, older conidia pushed aside by the younger to form a globular head enveloped in more or less slime; head falling to pieces easily. *Conidia* varying widely in size, form, and number of septa. *Microconidia* small and kidney or oval-shaped, with no septa, $3-4 \times 8-12\ \mu$ in diameter. *Macroconidia* typically sickle-shaped, with 1-4 septa when old; 2-septate spores $4 \times 11-20\ \mu$; 3-septate spores $4-5 \times 25-36\ \mu$; 4-septate spores $5 \times 38\ \mu$. *Conidial heads* globular, $30-35\ \mu$ in diameter.—Fig. 5.

This was the most abundant fungus found. It was rather uniformly distributed in all the plats and at all the depths. Ten or twelve mycelia of this were obtained on the plates to one of any other fungus.

ACROSTALAGMUS CINNABARINUS Cda.—RABEN-

HORST 37, 1²: fig. 340; OUDEMANS and KONING 29, pl. 6.—*Mycelium* orbicular, at first white, changing to orange or rose pink, be-

coming zoned with white margin and zones of light and dark pink or orange within; reverse color orange or pink; medium not colored. *Hyphae* branched, septate, $3-4\ \mu$ broad. *Conidiophores* upright, septate, twice verticillately branched, secondary branches $12\ \mu$ long, ninepin form, bearing at the end a globular head of conidia

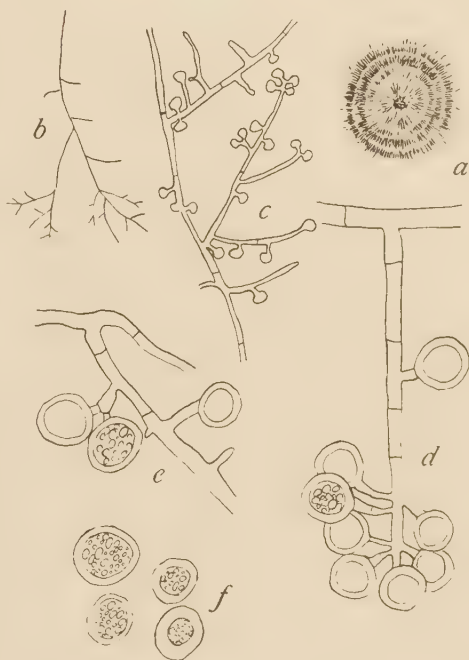


FIG. 4.—*Coccospora agricola*, n. sp.: a, habit sketch; b, branched hypha, $\times 80$; c, hyphae bearing conidia on simple side branches, $\times 180$; d, clustered conidiophores, $\times 380$; e, simple conidiophores bearing conidia, $\times 380$; f, conidia (chlamydo spores), $\times 380$.

enveloped in slime; heads $8-14\ \mu$ in diameter, easily falling apart. *Conidia* elliptical, $1.5-3 \times 4-6\ \mu$ in diameter.—Fig. 6, A.

This fungus, in most of the cultures, did not show the characteristic rose-red color, but rather a decided orange. However, several luxuriant plate cultures showing brilliant rose pink were developed on medium 1 by inoculation from an orange culture. Orange cultures were obtained again by inoculation from the rose pink.



FIG. 5.—*Fusarium* (sp. ?): a, b, hyphae bearing simple conidiophore, $\times 160$; c, same, $\times 380$; d, e, hyphae bearing conidial heads (microconidia), $\times 180$; f-j, stages in formation of conidial heads, $\times 380$; l, hyphae forming chlamydospores, $\times 180$; m, same, $\times 380$; n, microconidia, $\times 380$; o, macroconidia, $\times 380$.

PACHYBASIIUM HAMA-TUM (Bonord).—RABENHORST 37, 1²: fig. 311; ENGLER and PRANTL 36, 1²: fig. 440, A.—*Mycelium* at first white and scant, spreading to form a loose web, sometimes hardly visible on the substratum, soon forming irregular, white, woolly, hemispherical tufts 1-5 mm. broad, giving a scattered, botryoidal appearance; tufts at first white, becoming gray-green as spores form; reverse color the same; no coloration of the medium. *Hyphae* sparingly branched, hyaline, $3-5\ \mu$ broad. *Conidiophores*

rising upright from the hemispherical tufts, consisting of a twice or three times branched fertile portion and an elongated, terminal, sterile portion; fertile portion verticillately branched or forked to branches of the third order; end branches thick flask-shaped, $5-8\ \mu$ long, each having a sterigma-like end which bears a spore; sterile

ends with one or two short branches, septate, bent, and rising over the surface of the tufts giving a characteristic woolly appearance. *Conidia* single, pale green, gray-green in mass, $3-4 \times 5-7 \mu$ in diameter.—Fig. 7.

This description agrees well with RABENHORST, except that the sizes of the spores and of the flasklike end branches of the conidiophores are a little small. However, there seems little doubt that this is the same species.

ASPERGILLUS CALYPTRATUS Oudem.—OUDEMANS and KONING 29, pl. 13.—*Mycelium* floccose, spreading into a thick, white mat of irregular extent, little zonation, becomes avellaneous with age, green where spores are produced thickly changing to almost black; reverse side avellaneous to fulvous; no coloration of the medium. *Hyphae* richly branched, hyaline, septate, $3-4 \mu$ broad. *Conidiophores* upright or inclined, 0.3–0.4 mm. high, base hyaline and septate, swollen end elliptical or reverse pear-form. *Conidial fructification* long, cylindrical, dark green changing to black, $40-60 \times 100-200 \mu$ long. *Basidia* nearly cylindrical, pointed, densely packed, $6-8 \mu$ long. *Conidia* in long chains, globular, smooth, gray-green, $2-3 \mu$ in diameter.—Fig. 8, A.

ASPERGILLUS NIDULANS (Eidam).—ENGLER and PRANTL 36, 1: fig. 215.—*Mycelium* at first white, soon becoming chrome green with white or cream border, finally ochraceous to dirty green;

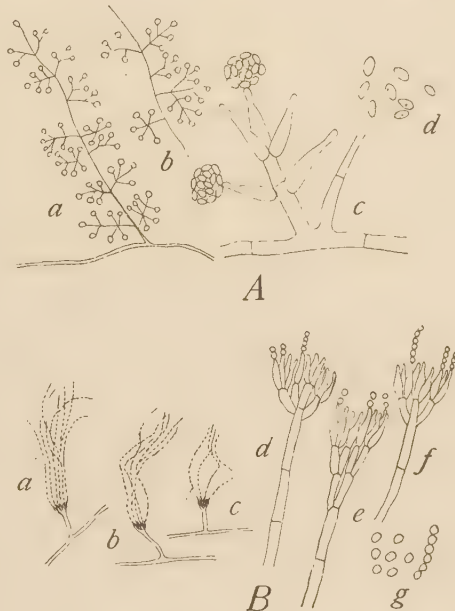


FIG. 6.—A, *Acrostalagmus cinnabarinus* Cda.: a, b, hyphae bearing conidiophores, $\times 80$; c, conidiophore with conidiiferous cells and conidial heads, $\times 380$; d, conidia, $\times 380$.

B, *Penicillium bicolor* Fries: a–c, conidial fructifications, $\times 80$; d–f, same, showing branching and conidiiferous cells, $\times 380$; g, conidia, $\times 380$.

surface having a botryoidal appearance especially on media 1 and 2; reverse color ochraceous; medium colored reddish brown. *Hyphae* much branched, septate, hyaline, $4-5\ \mu$ broad. *Conidiophores* upright, $0.1-0.3\ \text{mm.}$ long, septate; swollen end subglobose, $16-23\ \mu$ in diameter. *Conidial fructification* irregularly

globose, loose, dark green to black, $75-125\ \mu$ in diameter. Basidia once branched, primary branches club-shaped, about $5\ \mu$ long; secondary branches ninepin form, $7\ \mu$ long. *Conidia* in long chains, globose, $2.5-3\ \mu$ in diameter.—Fig. 8, B.

PENICILLIUM BICOLOR Fries.—*Mycelium* at first white, center becomes glaucous with white border, later develops a sulphur yellow outer zone which continues to form in the new growth, giving slight zonation, the whole finally changes through avellaneous to brown when old; yellow develops more brilliantly on ammonium nitrate media (nos. 1 and 2); less yellow and more glaucous on calcium nitrate medium (no. 3); reverse color fulvous to ferruginous; medium colored brown. *Hyphae*



FIG. 7.—*Pachybasium hamatum* (Bonord): a, habit sketch; b, fruiting tuft showing conidiophores and sterile ends, $\times 80$; d, branching conidiophore with whorls of conidiiferous cells, $\times 380$; c, fruiting hyphae with sterile end, $\times 180$; e, conidia, $\times 380$.

branched, many septa, much curved, $1.2-2\ \mu$ broad. *Conidiophores* upright, septate, hyaline, $200-300\ \mu$ long, $2-3\ \mu$ broad, branched 1-3 times at the top to form a compact fructification. *Conidial fructification* $100-200\ \mu$ long, consisting of branches to the second and at times to the third order (tertiary), the last bearing whorls of

conidiiferous cells on the end of which are long chains of conidia; fructification very compact; branches cylindrical, of about equal length, $8-10\ \mu$ long, $3\ \mu$ broad. *Conidiiferous cells* slender flask-form, slightly bent, $8-9\ \mu$ long. *Conidia* globular, nearly hyaline, pale green in mass, $2-3\ \mu$ in diameter.—Fig. 6, B.

This answers closely to the fungus described by OUDEMANS and quoted in RABENHORST under the name *bicolor*, as found in humous earth in Holland. I find nothing in THOM (33) that seems to correspond to it. It would appear to me that this form has decided specific characters, which distinguish it sharply from either *P. glaucum* Link or *P. crustaceum* L.

PENICILLIUM CANDIDUM Link.—*Mycelium* spreading, floccose, pure white slowly changing to glaucous, sometimes remaining perfectly white especially on media 1 and 2, finally gray to avellaneous in the oldest parts; reverse color yellowish to ochraceous or even ferruginous; medium uncolored. *Hyphae* branched, septate, about $3\ \mu$ broad. *Conidiophores* up to

$250\ \mu$ long, $3-4\ \mu$ broad, septate, hyaline, branched once or twice, often not at all, 1-5 conidiiferous cells at the end. *Conidiiferous cells* ninepin form, $9-12\ \mu$ long, $3-4\ \mu$ broad. *Conidial fructification* $100-150\ \mu$ long, often longer, loosely brushlike. *Conidia* smooth,



FIG. 8.—A, *Aspergillus calyptratus* Oudem.: a, b, conidiophores with conidial fructifications, $\times 80$; c, conidial head with sterigmata and conidial chains, $\times 380$; d, sterigmata, $\times 750$; e, conidia, $\times 380$.

B, *Aspergillus nidulans* (Eidam): a, conidiophores with conidial fructifications, $\times 80$; b, conidial head with sterigmata and conidial chains, $\times 380$; c, conidia, $\times 380$.

thin-walled, pale blue-green by transmitted light, $2 \times 3 \mu$ in diameter, varying to globose.—Fig. 9.

This description does not fit *P. candidum* perfectly. The spores of the form here described are oval and pale blue-green, while those of *P. candidum* are described as globular and white. However, these do vary to globular, although not typically. Again, the changing of the mycelium from white to gray-green and avellaneous is unlike *P. candidum*. However, this character varied on different media. On no. 3, the gray-green showed more decidedly than on 1 or 2. The white followed by avellaneous was more decided on no. 2. The color characters and also the spores seem to correspond more closely to the *P. camemberti* described by THOM. Differences in the other characters, however, would not at all favor this name. On the whole it has seemed best to call it *P. candidum*.



FIG. 9.—*Penicillium candidum* Link: a, b, conidial fructifications, $\times 80$; c-i, conidial fructifications with conidiiferous cells, $\times 380$; j, conidia, $\times 380$.

PENICILLIUM HUMICOLUM
Oudem.—OUDEMANS and
KONING 29, pl. 26.—This
fungus showed marked differ-
ences when grown on different
media. On media 1 and 2,
the mycelium was cream to
yellowish green, forming a
wrinkled, cheesy, superficial
crust which becomes dark
green (*atro-virens*) over por-
tions where spores form; mat of limited extent, usually orbicular;

no zonation, but with a narrow cream colored border; reverse color yellow (*melleus*); no coloration of the medium. When grown on medium 3, the mycelium formed no yellowish, wrinkled crust, but produced an orbicular, dark green mat, somewhat raised in the center and having a narrow white border; reverse color yellowish. *Hyphae* richly and irregularly branched, very irregular in form, showing constrictions and enlargements giving a gnarled appearance. many septa, short cells, contents foamy and vacuolated.

Conidiophores erect, little branched, hyaline, septate, up to $130\ \mu$ long, often very short, $3\text{--}4\ \mu$ broad. *Conidial fructification* long, narrow, compact, $75\text{--}120\ \mu$ long, $15\text{--}20\ \mu$ broad, branched trichotomously; primary branches tapering, bent, $8\text{--}10\ \mu$ long; secondary branches usually 3, at times 4, flask-form, $5\text{--}7\ \mu$ long. *Conidia* in long chains, hyaline, globular, $2\text{--}3\ \mu$ in diameter.—Fig. 10.

This comes very close to OUDEMANS' *P. humicolum*, although the breadth of the conidiophores and of the spores is slightly larger for the fungus here described. However, there was considerable variation in these sizes.

HORMODENDRON CLADOSPORIOIDES Sacc.—SORAUER 41, fig. 7.—*Mycelium* orbicular, smoky changing to black, slightly zoned with fringelike border; surface of mat becomes botryoidal and much wrinkled; reverse color black; medium not colored. *Hyphae* much branched, septate, somewhat gnarled, smoky to brown (umbrinus), $3\text{--}5\ \mu$ broad. *Conidiophores* a little branched at the top, septate, $75\text{--}100\ \mu$ long, $5\text{--}8\ \mu$ broad. *Conidial fructification* a dense, irregular mass of spores borne in chains with yeastlike branching. *Conidia* in branched chains, ends blunt or pointed, oval to elongate, $3\text{--}5 \times 6\text{--}15\ \mu$ in diameter.—Fig. 11.

MONILIA KONINGI Oudem.—OUDEMANS and KONING 29, pl. 21.—*Mycelium* white and finely floccose, forming a light brown



FIG. 10.—*Penicillium humicolum* Oudem.: *a, b*, conidial fructifications, $\times 80$; *c*, hyphae showing foamy, vacuolated condition, $\times 380$; *d*, gnarled hyphae; *e-h*, conidial fructification showing conidiiferous cells and conidial chains, $\times 380$; *i*, conidia, $\times 380$.

(light umbrinus) center which spreads and becomes somewhat zonate by varying densities of color, the whole finally becomes powdery brown; reverse color brown to ferruginous; no coloration of the medium. *Hyphae* branched, septate, hyaline, 4–5 μ broad. *Conidiophores* little differentiated from the mycelium, septate, once

or twice branched, the last branching often being di- or trichotomous, each division bearing a long conidiiferous cell. *Conidiiferous cells* flask-form, 30–40 μ long, often arising singly on simple side branches. *Conidial fructification* either simple or clustered, each flask-shaped cell bearing a long chain of conidia. *Conidia* in chains up to 30, often connected by a neck, terminal conidia often larger, globular to lemon-form, 6–9 μ in diameter, brown (light umbrinus), chains persistent.

—Fig. 12.



FIG. 11.—*Hormodendron cladosporioides* Sacc.: *a*, *b*, hyphae with conidiophores and conidial fructifications, $\times 180$; *c*, compact conidial fructification, $\times 180$; *d*, branching hyphae, $\times$ about 300; *e*, conidiophore and fructification, $\times$ about 300; *f*, same, $\times 380$; *g*, conidia, $\times 380$; *h*, habit sketch.

On the ammonium nitrate media (nos. 1 and 2), this fungus developed differently in its general appearance. The mycelium often formed a cream colored, cheesy, irregular crust with a loose network of hyphae

about the edges. This crust was finely zonate and had radiate grooves about the border. Later, spores formed thickly over the surface of the crust, giving a powdery, brown surface. Abundance of moisture favored this cheesy formation.

STYSANUS STEMONITES (Pers.).—RABENHORST 37, *t*²: fig. 337; OUDEMANS and KONING 29, *pl.* 29.—*Mycelium* at first white,

radiate, hemispherical, soon dark brown (dark umber) in the center, finally dusty brown with tufts of coremia, the whole having a narrow white border; reverse color rusty to dark brown; medium often changed to creamy, cheesy mass especially on ammonium nitrate media (nos. 1 and 2). *Hyphae* branched, hyaline, septate, $3.3\text{--}4.1\ \mu$ broad. *Coremia* in bushlike tufts $1\text{--}5\text{ mm.}$ in diameter; single coremia $1\text{--}3\text{ mm.}$ high, made up of a slender stalk and an expanded, cylindrical, brush-like head; head made up of long hyphae which send out short side branches each of which bears a single basidium or branches trichotomously, each division bearing a basidium-like cell. *Basidia* flask-shaped, $5\text{--}7\ \mu$ long, each bearing a long chain of spores. Spores are also produced in simple, umbellate clusters which are borne on short side branches. These clusters form especially at the edge of the medium. *Conidia* in long chains, oval to lemon-shaped, sometimes joined by a short neck, $3.5\text{--}4.5 \times 6\text{--}8\ \mu$ in diameter, pale bluish green when single, umber brown in mass.—Fig. 13.

***Trichoderma nigro-virens*, n. sp.**—*Mycelium* orbicular, at first white, spreading to form a thick, floccose mat, later developing successive concentric, dark green (*nigro-virens*) zones alternating with white, green zones preceded by white granular zonation, dark

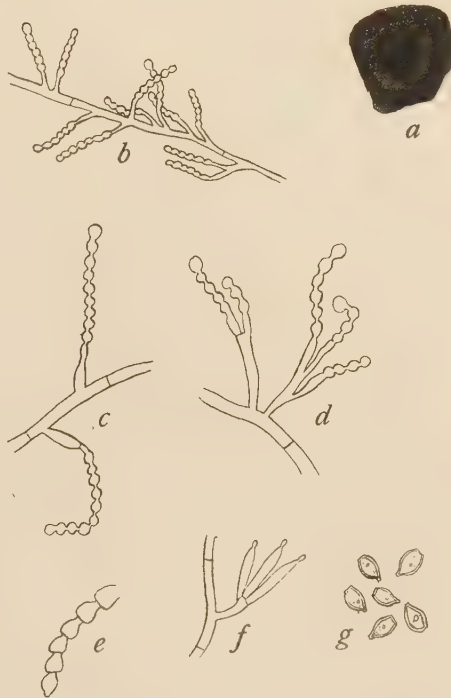


FIG. 12.—*Monilia Koningi* Oudem.: a, habit sketch; b, conidial fructification, $\times 80$; c, d, same, $\times 160$; e, chain of conidia, $\times 380$; f, conidiiferous cells, $\times 380$; g, mature spores, $\times 380$.

zones finally almost black, sometimes brown to olive green, giving a very characteristic and prominent black and white zonation with a broad white border; reverse color sulphur yellow to reddish brown on agar-ammonium nitrate medium; medium colored yellow. *Hyphae* richly branched, hyaline, septate, $1.7-2.4\ \mu$ broad.



FIG. 13.—*Stysanus stemonites* (Pers.): *a*, tuft of coremia, nat. size; *b*, tufts of coremia, $\times 2.5$; *c*, *d*, conidial fructifications, $\times 180$; *e*, conidiophores with conidiiferous cells and chains of conidia, $\times 380$; *f*, coremium, $\times 80$; *g*, conidia, $\times 380$.

Conidiophores branched, branches opposite or alternate, two or three times forked at the end, each end branch bearing 2 or 3 conidiiferous cells, each of which bears a conidial head at the tip. *Conidiiferous cells* long, tapering, abruptly pointed, $15-20\ \mu$ long, $3-4\ \mu$ broad. *Conidial heads* irregularly globular, $4-8\ \mu$ in diameter when young, massed together with age to form black, granular, masses $100-500\ \mu$ in diameter which form the black zones; conidiophores at last disappear, leaving only a mass of spores, whose arrangement often simulates chains. *Conidia* oval to fusiform, green by transmitted light, dark green to

black in mass, $3-5 \times 6-8\ \mu$ in diameter.—Fig. 14.

This was the third most abundant fungus found. It occurred in nearly all the plates. Its identification was especially difficult because of the rapidity with which the conidial heads massed together, making it hard to determine the character of the conidiophores. This was especially confusing on account of the chainlike arrangement of the spores in the granular masses. The black and white zonation was the most conspicuous character in the manner of growth. In some cultures, the spore masses fused together in the presence

of abundance of moisture to form a continuous, inky black, somewhat slimy surface.

TRICHODERMA KONINGI Oudem.—OUDEMANS and KONING 29, *pl.* 31.—*Mycelium* sparse, loose, spreading indefinitely, at first white and floccose, later bluish green and finally dark green (*atro-virens*) or olive green, spreading loosely and irregularly over the surface; reverse color olive green; no coloration of the medium. *Hyphae* richly branched, scattered, hyaline, septate, $2.5\text{--}3.5\ \mu$ broad, forming a loose web scarcely covering the medium. *Conidiophores* branched, opposite or alternate, twice or three times forked, each branch ending in 1–3 conidiiferous cells each of which bears a conidial head. *Conidiiferous cells* flask-shaped, $8\text{--}10\ \mu$ long, $3\text{--}4\ \mu$ broad. *Conidial heads* globular, without slime, $6\text{--}10\ \mu$ in diameter, easily breaking to pieces. *Conidia* pale green, transparent, elliptical varying to globular, $2.5\text{--}3 \times 3\text{--}4\ \mu$ in diameter.—Fig. 15.



FIG. 14.—*Trichoderma nigro-virens*, n. sp.: *a*, habit photograph; *b*, conidiophores, $\times 80$; *c*, conidiophores showing conidial heads, $\times 180$; *d*–*f*, conidiophores showing conidiiferous cells, $\times 380$; *i*, conidiiferous cell, $\times$ about 750; *g*, conidia, $\times 380$; *h*, germinating conidia.

Verticillium chlamydosporium, n. sp.—*Mycelium* orbicular, spreading into a thick mat with little zonation, at first white, later cream, and finally ochroleucous to ochraceous forming a firm crust; surface powdery with age; reverse color yellow (*flavus*) to orange; no coloration of the medium. *Hyphae* branched, septate, hyaline, $2\text{--}3.3\ \mu$ broad. *Conidiophores* upright, branched verticil-

lately; end branches $15-30\ \mu$ long, in whorls of 3 or 4, tapering to a knobbed end which bears a single spore. *Conidia* oval, hyaline, $2.2-3.5\ \mu$ in diameter, easily falling away. *Chlamydospore* formation especially common; chlamydospores multicellular, 4-9-celled, with granular contents, thick-walled, globular, $10-25\ \mu$ in diameter when mature, slightly lobed, borne on short side branches which are $15-30\ \mu$ long, very persistent.—Fig. 16.

This chlamydospore-formation is the most characteristic feature of this fungus. The older mycelium becomes a complete mass of these, matted in with hyphae, which at last disappear, leaving the chlamydospores as a creamy or ochraceous powder. The verticillate, conidial fructification forms more abundantly in the young mycelium at the edges of the culture, while later only chlamydospores are formed.

III. Assimilation of free nitrogen

GENERAL PLAN AND METHOD

In this part of the investigation an effort was first made to get some qualitative indication of nitrogen-fixation in a trial with all the fungi which had been isolated from the experimental plat. After this, it was planned to carry on further investigation with exact analytical methods, using any forms which gave any signs of such power. In carrying out this plan five separate investigations were made.

1. Culture solutions of 25 cc. each were prepared in large test tubes, which, after sterilization and inoculation by means of spores, were allowed to stand in the air protected only by germ-proof cotton stoppers.

2. A similar investigation was carried out, using 150 cc. Erlenmeyer flasks instead of test tubes, and using 50 cc. of the nutrient solution for each culture.

3. Cultures of four fungi, which gave the most hopeful indication of nitrogen, were prepared in an apparatus for aerating by means of air drawn through sodium hydroxide and concentrated sulphuric acid. Each culture contained 100 cc. of the culture solution.

4. Cultures similar to those in "3" were put under bell jars

which allowed entrance of air only through U-tubes containing concentrated sulphuric acid.

5. Cultures of one of these four fungi (*Myceliophthora sulphurea*) were made in Erlenmeyer flasks containing solutions with varying quantities of ammonium nitrate, to see whether nitrogen-fixation would not be possible when growth was started by small quantities of combined nitrogen. These cultures, each containing 50 cc. of the nutrient solution, were left exposed to the laboratory air, being closed only by cotton stoppers.

The media for all these cultures were made up by the use of ammonia-free water, as shown by tests with Nessler's reagent. This water, for some cultures, was prepared by a second distillation of distilled water, to which had been added a small quantity of sulphuric acid. The first part of the distillate was always discarded as an extra precaution. Some of the cultures were made with extra pure, so-called "conductivity water" obtained from the chemical laboratory of the University of Michigan.

The chemicals used in all the investigations were strictly C.P. Most of them were marked "Kahlbaum." Furthermore, they were all submitted to chemical analysis, which showed all to be nitrogen-free except the dextrose, which contained 0.25 mg. of nitrogen



FIG. 15.—*Trichoderma Koningi* Oudem.: a, hyphae with conidiophores, $\times 80$; b-d, conidiophores with conidiiferous cells and conidial heads, $\times 380$; e, conidia, $\times 380$.

per 1.0 gm. of dextrose. As pointed out later, this nitrogen did not appear to be in a form which was available to the fungi.

In every investigation, all the glassware for the cultures was cleaned with the greatest care by being placed for 6-24 hours in a mixture of potassium dichromate and concentrated sulphuric acid.

All rubber for connections was new and was first boiled in a 10 per cent solution of sodium hydroxide until perfectly clean.

Sterilizations were carried out in an autoclave raised to 150° C. at least 20 minutes. It was found that temperatures much above this were likely to cause some decomposition of the dextrose, giving a brown coloration of the medium (DUGGAR 32, p. 20).

Inoculations were made in all cases by introducing, with a sterilized platinum loop, a small quantity of spores from a pure culture into about 10 cc. of sterilized distilled water in a small test tube. Then from this one or two drops were transferred to the sterilized culture flasks by means of sterilized pipettes



FIG. 16.—*Verticillium chlamydosporium*, n. sp.: a, conidiophores, $\times 80$; b, portion of conidiophore with conidiiferous cells, $\times 380$; c, e, chlamydospores, $\times 380$; d, young chlamydospores, $\times 380$; f, conidia, $\times 380$.

held in a corked and germ-protected beaker. That the cultures were inoculated with viable spores was shown by controls, in which spores from the same source were inoculated into nitrogen-containing media. Such controls all showed vigorous and characteristic growth, with the exception of the first investigation as explained hereafter. Furthermore, some growth took place in all

the nitrogen-free cultures, and microscopic examination established the purity of these growths.

The fungi tested in this series of investigations include all those previously given in the complete list, excepting *Mucor stolonifer*, *Penicillium glaucum*, *P. bicolor*, and *Trichoderma Koningi*.

In the last four investigations, cultures were submitted to the most careful chemical analyses to determine the quantity of nitrogen in the culture at the beginning and at the end of the growth period. Proper controls were used in each case.

In all the five investigations above mentioned, the results were entirely negative for all the 15 fungi tried, under the conditions of the investigations. The highest amount of nitrogen found in any culture in a nitrogen-free medium, after the method of analysis had been mastered, was 0.46 mg. in a 50 cc. culture. A repetition of this culture under essentially the same conditions showed only 0.11 mg. gain. One other result of 0.31 mg. gain was obtained, although the duplicate culture showed a gain of but 0.03 mg. Aside from these results mentioned, all the values for the nitrogen gain are well within the limits of error of the method. This error is somewhere about 0.14 or 0.21 mg. Indeed, it is doubtful if results can always be obtained as accurately as this. In the cultures of investigation V, containing ammonium nitrate, the highest nitrogen gain was 0.27 mg. in a 50 cc. culture which contained at the beginning 0.22 mg. fixed nitrogen.

CHEMICAL ANALYSES

For the chemical analyses, different modifications of the Kjeldahl method were employed. In general, these methods were in accordance with the official methods of the Association of Official Agricultural Chemists of the United States. For nitrate-free media, the Gunning method was used, modified by the use of copper sulphate, according to the investigations by HIBBARD (43) and in accord with the method used by TERNETZ (19; see HOPPE-SEYLER 44). For the analysis of media containing ammonium nitrate, the "Official Gunning modification to include the nitrogen of nitrates" was used, modified by the use of copper sulphate as in

the "nitrate-free method." The details for these methods were as follows:

1. *For the nitrate-free method.*—The material to be analyzed was first placed in the Kjeldahl flask. This material was either in the form of a solid, or in the case of solutions was evaporated nearly to dryness as hereafter described. Next, 10 gm. of a mixture of potassium sulphate and copper sulphate were added to the flask. The proportion of these which gave the best results was 9.2 parts of K_2SO_4 to 0.8 parts of $CuSO_4$. Then 25 cc. of sulphuric acid was added, after which gentle heat was applied. This was increased gradually to strong heat and this was continued for 15–30 minutes after clearing. The contents of the Kjeldahl flask were now rinsed into a 1000 cc. distillation flask and diluted with 240 cc. ammonia-free water. The whole was made alkaline by the addition of 120 cc. of a strong solution of sodium hydroxide (40 gm. pure sticks to 120 cc. ammonia-free water). Two small pieces of stick zinc (granulated zinc caused too much foaming) were now added, and then heat was applied until about 250 cc. of the distillate had passed over into the standard acid. The volume of the standard acid was in most cases 20 cc.

2. *Method in presence of nitrates.*—The material was added to the digestion flask and when this was thoroughly cool, 30 cc. of a mixture of concentrated sulphuric acid and salicylic acid were added. In most cases these two, previously mixed, were added in the proportion of 1 gm. salicylic to 30 cc. sulphuric; but when large quantities of nitrate were present, 1 gm. of salicylic was used to 25 cc. sulphuric. The flask was now allowed to stand 5–10 minutes with frequent shakings, and then 5 gm. sodium thiosulphate were slowly added and well mixed. Heat was then slowly applied for about 5 minutes or until white fumes began to appear, after which the whole was cooled for 5–10 minutes. Next, 10 gm. of a mixture of potassium sulphate and copper sulphate were added; gentle heat was applied till all foaming ceased, and lastly, strong heat was applied till 15–30 minutes beyond clearing. Distillation was carried out as in the nitrate-free method.

The titration method was used throughout for determining the quantity of ammonia in the distillate. For very small quantities

of ammonia, as in nitrogen-free cultures, the distillate was passed into N/20 sulphuric acid, which was then titrated by the use of N/20 sodium hydroxide. In some cases with larger nitrogen content, N/10 or even N/5 solution of the acid was used. Ammonia-free water was employed for all the distillations. The distilled water of the laboratory was found to contain 0.21 mg. of nitrogen in 300 cc. of the water. The ammonia-free water for this was prepared by the evaporation of distilled water to one-half its original volume after the addition of a small piece of sodium hydroxide.

After the titration, the quantity of nitrogen was calculated as follows: 1 cc. N/10 sulphuric acid is equivalent to 1 cc. N/10 ammonia (NH_3); 1 cc. N/10 ammonia contains 1.404 mg. of nitrogen; then the nitrogen present is equal to the number of cc. of N/10 acid used times 1.404 mg.

All chemicals used in the analyses were the purest that could be obtained. The sulphuric acid was marked "Baker and Adamson, standard purity." At the same time, the purity of the chemicals was tested by a blank in which only the chemicals were used. This showed the chemicals used in the nitrate-free method to contain 0.42 mg. of nitrogen, and in the nitrate method 0.56 mg. The amount of the proper blank was subtracted in each case from the total nitrogen of the determination. The filters used in separating the mycelium from each of the cultures were analyzed and found to be nitrogen-free.

The apparatus employed for the digestion consisted of 500 cc. Kjeldahl flasks of the oval form, each provided with a straight bulb tube, which was inserted in the neck of the flask to prevent loss of sulphuric acid. The distillation was carried out in 500 cc. round bottom Jena flasks. These were connected with Kjeldahl bulbs by means of rubber stoppers, and the bulbs led into glass condensers which were placed in an upright position. The distillate was collected in a 500 cc. Erlenmeyer flask. The burettes used for the titrations were the most carefully standardized, with enamel backs and a blue line for accurate readings. All apparatus and utensils were washed with the greatest care after use each time. In addition, they were rinsed at least twice with distilled water, after which they were either inverted or covered.

The standard solutions were standardized in two ways. First, by titration of the alkali against standard succinic acid, which was prepared by dissolving 5.903 gm. of C.P. salt in 1000 gm. of distilled water. Second, these results were confirmed by reference to standard hydrochloric acid whose absolute strength had been determined by precipitation with silver nitrate and weighing of the silver chloride. The sodium hydroxide was found to gain strength by standing in glass, so this was checked every few days against the standard acid.

Methyl red (45) was employed as the indicator for titrating the distillate. This was found to give a much sharper and more accurate end reaction than either cochineal or methyl orange. The whole of the distillate was used for titration. The results were found much less accurate when only part was used and computation made for the whole. In standardizing solutions, phenolphthalein was usually used as indicator, although with the strength of acids employed, the methyl red was found to be equally accurate.

Culture solutions were evaporated over a water bath, down to about 10 cc., before adding them to the digestion flask. Previous to the evaporation, 5 drops of concentrated sulphuric acid were added to fix any free nitrogen present. This precaution applied especially to the cultures left exposed to laboratory air. In the analyses when ammonium nitrate was present, this evaporation was found to be especially necessary, since the heat resulting from the mixture of the acid and water caused a loss of nitrogen in the form of nitric acid or oxides of nitrogen. To avoid this, extra precautions were taken. After the evaporation had been carried to about 10 cc. in the usual way, this quantity was transferred to a Kjeldahl flask with two or three small rinsings, and then the evaporation was carried nearly to dryness in this flask over a water bath. It was also found necessary to have the contents of the flask perfectly cool before adding the mixture of sulphuric and salicylic acids. To accomplish this, the flask was cooled in ice water and then held in ice water while adding the mixture of acids. As a still further precaution, about 0.5 gm. of salicylic acid was added to the material to be analyzed and thoroughly mixed before adding the sulphuric-salicylic mixture. In using the ammonium nitrate

in the form of the dry salt for the test analyses, it was found necessary to have the salt thoroughly powdered before adding the sulphuric-salicylic mixture. In nearly all cases the analyses were carried through in duplicate.

By both methods of analysis used, a series was carried through with some substance of known composition and purity, in order to make sure of accurate results from the method. Asparagin was used for the nitrate-free method, while ammonium nitrate was employed for the method including nitrates. For all small amounts of asparagin, a suitably large quantity of the pure salt was carefully weighed on an analytical balance and then dissolved in a quantity of water measured up in a volumetric flask. For each analysis, the desired quantity of this solution was measured off into an evaporating dish by use of an accurate burette. The solution was then evaporated down to 5-7 cc., and was then poured into the Kjeldahl flask, together with two or three small rinsings. The following tabulation gives the results of these analyses in comparative view:

TABLE IV

Blank determination gave N 0.42 mg.; calculated per cent of N in asparagin, 18.70; solution 1, 80 mg. asparagin dissolved in 200 cc. distilled water; solution 2, 300 mg. asparagin dissolved in 1000 cc. distilled water.

No.	Material used	N obtained less blank mg. <i>a</i>	Calculated amount mg. <i>b</i>	Difference (<i>b-a</i>) mg. <i>c</i>	Percentage obtained <i>d</i>	Variation from calc. percentage <i>e</i>
1	100 mg. dry salt.....	17.97	18.70	0.73	17.97	-0.73
2	100 " " ".....	18.11	18.70	0.59	18.11	-0.59
3	100 " " ".....	18.04	18.70	0.66	18.04	-0.66
4	50 " " ".....	8.98	9.35	0.37	17.96	-0.74
5	50 " " ".....	8.98	9.35	0.37	17.96	-0.74
6	50 " " ".....	8.92	9.35	0.43	17.83	-0.87
7	20 " " ".....	3.79	3.74	0.05	18.90	0.20
8	20 " (50 cc. sol. 1)...	3.65	3.74	0.09	18.25	-0.45
9	15 " (50 cc. sol. 2)...	2.67	2.80	0.13	17.80	-0.90
10	15 " (duplicate of 9)...	2.70	2.80	0.10	18.00	-0.70
11	3 " (10 cc. sol. 2)...	0.53	0.56	0.03		
12	3 " (duplicate of 11)...	0.49	0.56	0.07		

The similar analyses were not run in duplicate except as indicated. It will be seen that the greatest variation occurs in the case of no. 7, where a relatively small quantity of dry salt was used.

It seems probable that this is accounted for by the inaccuracy of weighing and transferring so small a quantity, an error which becomes relatively very great in so small an amount. If we eliminate this result, it is quite evident from the table that the nitrogen content of asparagin as shown by the analyses is a little lower than the calculated amount. The difference is very small and without much doubt is attributable to slight impurity of the salt. Considering the 50 and 100 mg. analyses only, we see a variation in the different analyses, not made at the same time, of only 0.28 of 1 per cent. This seems a good degree of accuracy for such determinations. Of course, the per cent of error in quantities as small as 3 mg. of the asparagin salt would have little significance. Considering the absolute variation of the analyses, including 20 mg. and below (not including no. 7), we see only 0.1 mg. difference between the maximum and minimum variation from calculated results. This is true even with slightly impure asparagin. These quantities of nitrogen are, it may be observed, larger than those dealt with in the nitrogen-free cultures. It would seem safe to say then that the limit of error of the method was not far above 0.1 mg. in each determination. After considerable experience with determinations under different conditions, it is believed that this limit might run as high as 0.3 mg. in a long series of determinations.

The weighing up of the mycelium in the cultures was done, as were all other quantitative weighings, on an exact Becker analytical balance which weighed accurately to 0.2 mg. The filters were first dried in an oven at 100° C. until they showed a constant weight (about 6 hours). The culture was then filtered and the filter containing the mycelium was dried to a constant weight. Glass-stoppered weighing bottles were used for all this weighing and drying. As previously stated, the filters were found by a special analysis to be nitrogen-free.

Analyses were also made to test the accuracy of the method when nitrates were present. First, an analysis was made of 400 mg. of dry ammonium nitrate. This was the amount contained in the nitrate cultures of the highest concentration. Next, an analysis was made of the original culture solution from which all the cul-

tures of investigation V were made. This solution contained, in addition to the usual culture constituents, 400 mg. of ammonium nitrate. From this solution 50 cc. were accurately measured from a burette and then diluted to 250 cc. in a volumetric flask. Then 50 cc. of this solution was taken for analysis. This contained 80 mg. of ammonium nitrate. The results of these analyses are given in table V.

TABLE V

Blank by nitrate method, 0.56 mg. N; control with other constituents of the medium, but no ammonium nitrate, 0.70 mg. N; calculated percentage of N in ammonium nitrate, 35.04.

No.	Material	N obtained less blank mg. <i>a</i>	Calculated amount mg. <i>b</i>	Difference (<i>b</i> — <i>a</i>) mg. <i>c</i>	Percentage N obtained <i>d</i>	Deviation from calculated percentage <i>e</i>
1	400 mg. dry NH ₄ NO ₃	138.6	140.1	1.5	34.6	0.4
2	400 mg. (duplicate)	139.2	140.1	0.9	34.8	0.2
		N obtained less blank and control				
3	80 mg. (50 cc. of solu- tion)	27.79	28.04	0.25	34.7	0.3
4	80 mg. (duplicate)	27.79	28.04	0.25	34.7	0.3

These results seem to show that the method is sufficiently accurate for detecting any appreciable gain in nitrogen in the cultures containing ammonium nitrate.

INVESTIGATION I

As previously stated, cultures were made in large test tubes, each containing 25 cc. of the culture medium. This medium had the following composition:

- Water (ammonia-free) 1000.00 cc.
- Rock candy (pure), M/20 17.107 gm.
- Monopotassium phosphate, M/100 1.361 gm.
- Magnesium sulphate, M/500 0.241 gm.
- Calcium carbonate, M/1000 0.100 gm.

For controls, ammonium nitrate was added to the above as follows:

- Ammonium nitrate 0.360 gm.

The following 11 different species were used for the inoculations:
Mucor ambiguus, *Myceliophthora sulphurea*, *Coccospora agricola*,

Fusarium (sp. ?), *Acrostalagmus cinnabarinus*, *Pachybasium hamatum*, *Aspergillus calypttratus*, *Hormodendron cladosporioides*, *Monilia Koningi*, *Stysanus stemonites*, and *Trichoderma nigro-virens*. Four inoculations were made with each fungus, two in N-free cultures and two in ammonium nitrate cultures. This gave duplicates of all cultures. Growth took place in all of these, except those of *Stysanus* and *Hormodendron*. One of the duplicates for each of these failed to show any germination, and evidence was obtained later that the spores were no longer viable. In one or two cases there were doubts about the purity of cultures. On these accounts, it was decided to make another trial of the whole set. This result should be noted, however, that while a visible amount of growth took place in all these cultures, the amount was extremely small in all N-free cultures, and furthermore, it all took place within two or three weeks, after which no growth was to be observed, although observations were continued for over three months.

INVESTIGATION II

The second investigation was carried out along the same general lines as the first, but with several modifications. First, Erlenmeyer flasks of about 150 cc. capacity were substituted for the test tubes. In each flask was placed 50 cc. of the culture medium instead of 25 cc. as before. Second, the culture solution was modified, using a higher percentage of phosphate and nearly doubling the amount of sugar, which was supplied this time in the form of dextrose. The full formula was as follows:

Ammonia-free water.....	1000.00 cc.
Dextrose.....	30.00 gm.
Monopotassium phosphate.....	2.00 gm.
Magnesium sulphate.....	0.20 gm.
Calcium carbonate.....	0.10 gm.
Sodium chloride.....	0.02 gm.
For controls:	
Ammonium nitrate	2.00 gm.

This was a medium which had been found well adapted to luxuriance of growth in agar and gelatin cultures. Third, 14 species of fungi were used for the inoculations, including all in the complete

list except *Mucor stolonifer*, *Penicillium glaucum*, *P. bicolor*, *Stysanus stemonites*, and *Trichoderma Koningi*. New cultures had been made of all of these and it was made certain that the spores were all viable and the cultures all pure. Inoculations were again in duplicate, giving four cultures of each fungus, two in N-free media and two in ammonium nitrate media. All the cultures were placed in the laboratory, protected from the air only by cotton stoppers. Observations were made once a week from January 3 to March 11, about 68 days.

Good growth took place in all the cultures and there were good indications that all were pure according to the inoculations. This was shown first by the fact that the duplicates in every case were very closely similar. Second, each culture showed the peculiar culture characters belonging to the species, such as coloring the medium pink in the case of *Fusarium*, or the black mycelium in the case of *Hormodendron*. Third, the four that were afterward analyzed were submitted to microscopic examination, which showed characteristic spores and fructifications, although spores were only meagerly produced in the N-free cultures. The amount of growth in the N-free cultures as compared with that in the ammonium nitrate cultures, as it appeared to the eye, is shown in fig. 17. The mycelium in the former was very loose and flocculent and almost wholly submerged. To the eye, it appeared to spread through two-thirds of the liquid in some cases, but as will be seen in the analytical results, when dried its weight was extremely small. It is worthy of note also that this growth all took place within the first 2 or 3 weeks, after which it ceased entirely. The growth in the ammonium nitrate medium was very abundant. The mycelium soon reached the surface, where spores began to form abundantly. The growth continued for many weeks and finally resulted in a thick felt over the surface and a mass of mycelium filling the liquid.

Five of these cultures which gave signs of the largest growth in the N-free solutions were now taken for analytical examinations. The mycelium of each was collected on a dried filter and dried to a constant weight. The filtrate was evaporated as previously described and analyses were made of both mycelium and filtrate.

A control was also analyzed to show the amount of nitrogen present. The control was treated exactly like the others except that it was sterilized immediately after inoculation.

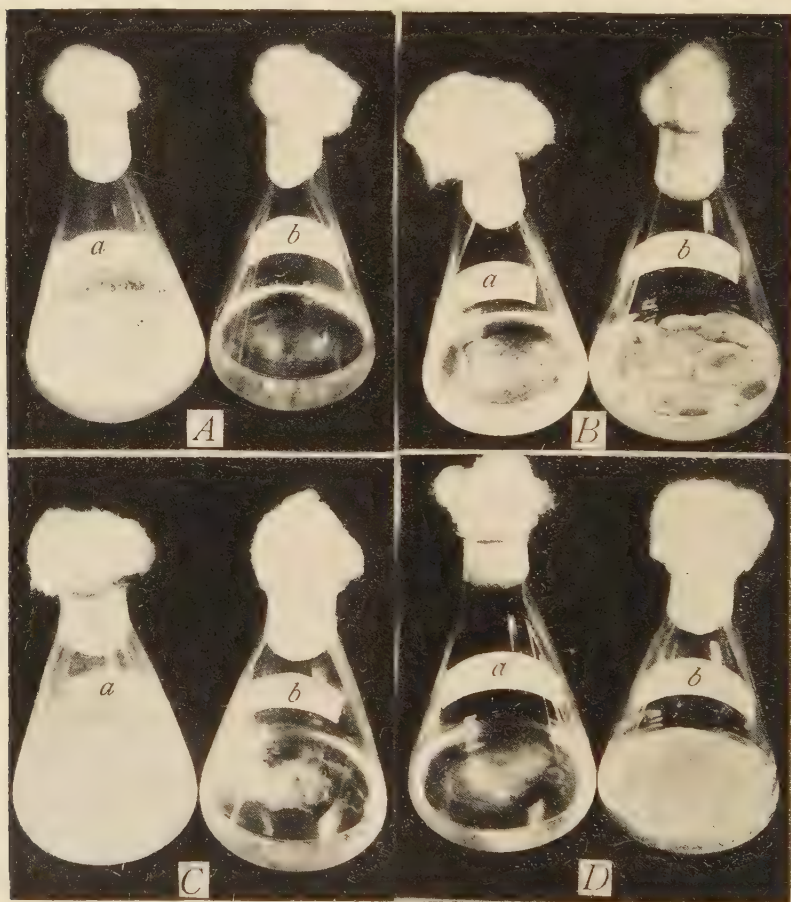


FIG. 17.—A, *Fusarium* (sp. ?): a, on ammonium nitrate medium; b, on nitrogen-free medium.

B, *Acrostalagmus cinnabarinus*: a, on nitrogen-free medium; b, on ammonium nitrate medium.

C, *Verticillium chlamydosporium*: a, on ammonium nitrate medium; b, on nitrogen-free medium.

D, *Pachybasium hamatum*: a, on nitrogen-free medium; b, on ammonium nitrate medium.

Table VI shows the results of this set of analyses. The nitrogen of a blank determination has been subtracted in each case from the total nitrogen obtained.

TABLE VI
DEVELOPMENT PERIOD 68 DAYS

No.	Fungus	Dry wt. of mycelium mg. <i>a</i>	N of mycelium mg. <i>b</i>	N of filtrate mg. <i>c</i>	Total N of culture mg. <i>d</i>	N fixed (total control) mg. <i>e</i>
1	Control inoculated with Myceliophthora			0.49	0.49	
2	Control inoculated with Hormodendron			0.42	0.42	
	Av. for control			0.45	0.45	
3	Myceliophthora	6.0	0.49	0.42	0.91	0.46
4	Hormodendron	7.1	0.42	0.28	0.70	0.25
5	Hormodendron (dup.)	9.4	0.07	0.21	0.28	-0.17
6	Pachybasium	5.0	0.14	0.42	0.56	0.11
7	Pachybasium (dup.)	5.6	0.07	0.42	0.49	0.04
8	Acrostalagmus	8.6	0.28	0.14	0.42	-0.03
9	Acrostalagmus (dup.)	9.0	0.21	0.28	0.49	0.04
10	Fusarium	6.0	0.07	0.35	0.42	-0.03
11	Fusarium (dup.)	4.2	0.07	0.07	0.14	-0.31

From later work, it was believed that the dry weights of mycelium, given in column *a* of the table above, were a little too large. The reason for this seemed to be that the mycelia were not washed quite long enough to remove every trace of dextrose. Again, the nitrogen fixed, as indicated in column *e*, was high enough for nos. 3 and 4 to raise the question whether these two fungi might possibly have a little nitrogen-fixing power. To test these points further, cultures of these two fungi were run at the same time with later investigations, extending from April 17 to June 26. These cultures were treated exactly as in the above series, except that the phosphate content was increased to 3.0 gm., and the dextrose content to 45.0 gm. in 1000 cc. of the solution. This gave a higher nitrogen content for the controls, as well as for the filtrates of the cultures, since the glucose was found to contain some nitrogen. In washing the mycelia, 200 cc. of water was used instead of 100 cc. as in the earlier work. Table VII gives the results of these tests.

It is seen from this table that the amount of nitrogen gain in this case is well within the limit of error. The slightly higher value in the earlier work is believed to be due to less skilful use of the analytical methods. In harmony with this idea is the fact that *Myceliophthora* was the first one of the set which was analyzed. The results on the duplicate were thrown out altogether as worth-

TABLE VII

No.	Fungus	Dry wt. of mycelium mg. <i>a</i>	N of mycelium mg. <i>b</i>	N of filtrate mg. <i>c</i>	Total N of culture mg. <i>d</i>	N fixed (total control) mg.
12	Control.			0.63	0.63	
13	Control (dup.)			0.70	0.70	
	Av. control.			0.66	0.66	
14	<i>Myceliophthora</i>	2.8	0.07	0.70	0.77	0.11
15	<i>Hormodendron</i>	3.0	0.14	0.56	0.70	0.04

less, due to a bad frothing in the distillation which evidently carried over some of the alkali. This difficulty was relieved by exchanging the 500 cc. distillation flasks for the 1000 cc. size, and using only stick zinc instead of granulated, after which no further difficulty of this kind was experienced. These results therefore give no foundation for nitrogen-fixation in these fungi, under the conditions of the investigation.

INVESTIGATION III

This investigation proceeded to work further with these same fungi, omitting *Acrostalagmus*, in nitrogen-free media, according to the method employed by TERNETZ (19), PENNINGTON (25), and others, using an apparatus for aerating the cultures by air drawn through strong potassium hydroxide and concentrated sulphuric acid. The apparatus is shown just as it was used in fig. 18. It consisted of six 500 cc. Erlenmeyer flasks connected up in sets of three in such a way that the air to each set passed over the following series: a U-tube of pumice, saturated with strong NaOH; a U-tube containing strong H₂SO₄; a wash bottle $\frac{1}{3}$ filled with sterilized, distilled water; a tube 2 cm. in diameter containing a

germ-proof cotton stopper. The tube leading from the last divided into three, one entering each culture flask and dipping below the liquid. Each tube before entering the culture flask contained a bulb 2 cm. in diameter, which was also filled with cotton loosely packed. An exit tube passed out of each flask. The three from

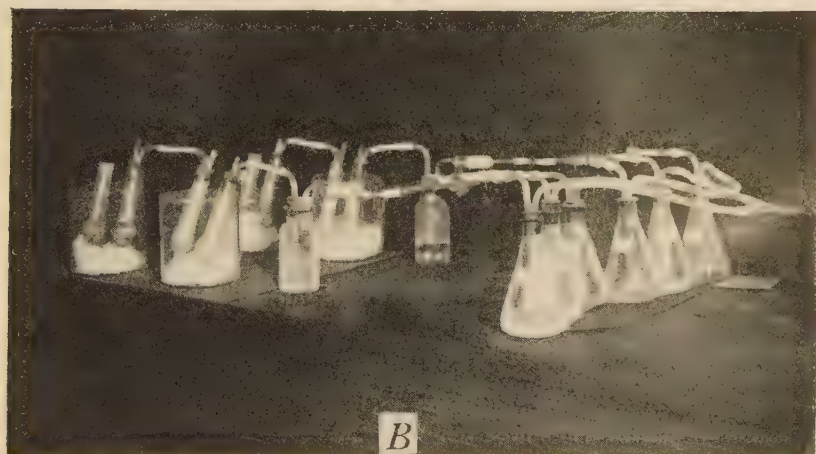
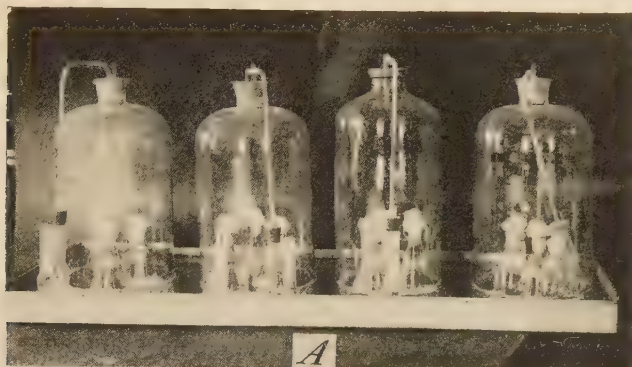


FIG. 18.—Apparatus for investigations IV (A) and III (B)

each set united into one, and these common tubes from each set united to form one tube which was connected to a filter pump. By the operation of this pump a slow stream of air was kept bubbling through all the cultures of both sets during the entire culture period. By means of clamps this stream was kept quite uniform

in all the flasks. Connections were made by means of new rubber stoppers and rubber tubing, which had been boiled in a 10 per cent solution of NaOH until perfectly clean. In addition, rubber stopper connections were sealed with paraffin. There was every indication that all the connections were air tight. The medium used for these cultures was made up as follows:

Conductivity water (ammonia-free).....	1000.00 cc.
Dextrose.....	45.00 gm.
Monopotassium phosphate.....	3.00 gm.
Magnesium sulphate.....	0.20 gm.
Calcium carbonate.....	0.10 gm.
Sodium chloride.....	0.02 gm.

Of this medium 100 cc. was supplied to each flask and then all apparatus except the U-tubes was sterilized in the autoclave at 115° C. Open ends were plugged with cotton during the sterilization and then connections were made as quickly as possible after removing these. Inoculations were made with the usual precautions, giving one culture for each of the four forms: *Hormodendron*, *Myceliophthora*, *Fusarium*, and *Pachybasium*. Two controls were prepared, one inoculated with *Myceliophthora* and the other with *Hormodendron*, and both sterilized immediately after inoculation. The latter of these controls became contaminated during the culture period and is therefore not considered in the analytical results. These cultures were allowed to develop from February 24 to May 4.

A perceptible growth occurred in all except the controls and examination, including microscopic, showed the cultures to be pure. The growth, while perceptible, was very scant. To the eye it appeared a little less than in those of investigation II, which stood in laboratory air. At the end of the development period previously mentioned, the cultures were disconnected and all sterilized in the autoclave. As soon as possible analyses were made as before, for mycelium and filtrate separately. The one uncontaminated control was also analyzed. The results are shown in table VIII.

From table VIII it is seen that no indication whatever is shown of nitrogen-fixation. The values for all the cultures are well within the limit of error of the analytical method.

In table VIII are also included some analyses of the medium to determine whether it took up an appreciable amount of fixed nitrogen by remaining exposed to the air of the laboratory. One culture was analyzed immediately after being made up, another after being kept during the last development period (70 days) under a bell jar, to which air could enter only over a U-tube containing concentrated H_2SO_4 . A third was exposed for 70 days to laboratory air, which could enter freely through the cotton stopper. The analyses of these cultures show clearly that no fixed nitrogen, which was detectible by the method of analysis, was taken up from the air.

TABLE VIII
DEVELOPMENT PERIOD 70 DAYS

No.	Fungus	Dry wt. of mycelium mg. <i>a</i>	N of mycelium mg. <i>b</i>	N of filtrate mg. <i>c</i>	Total N of culture mg. <i>d</i>	N fixation (total control) mg. <i>e</i>
1	Control.....				1.18	
2	Myceliophthora.....	3.0	0.07	1.26	1.33*	0.15
3	Hormodendron.....	1.3	0.07	1.19	1.26	0.08
4	Pachybasium.....	3.1	0.00	1.12	1.12	-0.06
5	Fusarium.....	1.5	0.07	1.26	1.33	0.15
6	Medium immediately after making up.....				1.40	
7	Medium after standing exposed 70 days.....				1.40	
8	Medium under bell jar 70 days.....				1.50	

Table VIII shows a further point of some interest, viz., that the nitrogen which was shown to be present in the dextrose used is not available for the fungi, since approximately the same quantity is shown in the filtrates in which fungi have been cultivated as in the medium itself before inoculation. Furthermore, the spores introduced in inoculation do not add an appreciable quantity of nitrogen.

INVESTIGATION IV

This was carried out with cultures exactly similar to those in the previous investigation; but instead of being aerated, they were kept under bell jars from which all air was excluded, except that entering through a U-tube containing concentrated sulphuric

acid (fig. 18). The bell jars were sealed by means of vaseline to ground glass plates and water was kept in the pan enough to cover the lower edge of the bell jars at all times. These all developed some growth, not perceptibly different from the aerated cultures. Each fungus developed its specific characters. Analysis was made of only one of these cultures, but the dry weight of the mycelium was found for all. A control was also analyzed. The development period was the same as for those in investigation III (70 days). A comparison was also made in this investigation between the growth in ammonia-free water, prepared by another distillation of distilled water acidulated with sulphuric acid, and in the "conductivity water" obtained from the chemical laboratory. As seen from the dry weights in the table, no perceptible difference could be detected. Very little growth occurred in either. The data are given in table IX.

It is evident, by a glance at table IX, that these cultures showed no evidence of nitrogen-fixing power. The dry weights of the last four are too small to give any expectation of finding nitrogen by analysis. Repeated analyses of such amounts have shown no appreciable nitrogen present. Moreover, the one analyzed (no. 1) fully confirms this idea.

INVESTIGATION V

An attempt was made in this investigation to see whether fungi, which were supplied with sufficient combined nitrogen to start a good growth, would not under such conditions show a power to fix some free nitrogen. This was especially desirable, since the results of BERTHELOT (13), PURIEWITSCH (16), and LATHAM (20) have shown such decided nitrogen-fixation in such solutions.

The medium used was exactly the same as that employed in the last two investigations, except that varying amounts of ammonium nitrate were added. These solutions were made as follows: First, Erlenmeyer flasks of 150 cc. capacity were selected and cleaned ready for the solutions. Then a solution exactly like that used in investigation III was made up, using only half as much water, thus giving a solution of double the strength of all the constituents. This was called solution A. Then a solution of ammonium nitrate

was made in which exactly 16 gm. of the C.P. salt were dissolved in ammonium-free water. The salt for this was of course weighed out with the greatest accuracy on the analytical balance. The solution was then made up to exactly 1000 cc. in a volumetric flask, and was called solution *B*. Cultures were all made in duplicate. First, 25 cc. from solution *A* and 25 cc. from solution *B* were drawn off, by the use of an accurate burette, into duplicate flasks. This gave 50 cc. cultures, each of which contained exactly 0.40 gm. of pure ammonium nitrate, or approximately an M/10 solution. Then 50 cc. of solution *A* were accurately drawn off into a volumetric flask of 250 cc. capacity. This flask was then filled to the mark with ammonia-free water, thus giving a solution

TABLE IX
CONTROL, 100 CC., STERILIZED AFTER INOCULATION, 1.32 MG. N

No.	Name of fungus	Dry wt. of mycelium mg. <i>a</i>	N of mycelium mg. <i>b</i>	N of filtrate less control mg. <i>c</i>	Total N (<i>b-c</i>) mg. <i>d</i>	Gain mg. <i>e</i>
1	Myceliophthora.....	3.0	0.07	0.08	0.15	0.15
2	Hormodendron (conductivity water).....	1.6	Not analyzed			
3	Fusarium (conductivity water).....	1.2				
4	Fusarium (non-conductivity water).....	1.0				
5	Pachybasium.....	1.6				

of $\frac{1}{5}$ the strength of solution *B*. Two culture solutions were now made by accurately drawing off 25 cc. of this diluted solution of ammonium nitrate and 25 cc. of solution *A* into culture flasks. This gave an M/50 solution of ammonium nitrate. By the continuation of this dilution process, further culture solutions were made containing M/250, M/1250, and M/6250 respectively of ammonium nitrate, all solutions having the same quantities of the other constituents as in investigation III. This may all be seen in brief in table X.

These cultures were sterilized and inoculated in the usual way and then allowed to stand in the laboratory without special protection from the air. During the final sterilization, a slight change

took place in the M/10 and in the M/50 media, a change which was more marked in the former. The cloudiness due to the undissolved calcium carbonate had entirely disappeared and the medium had become slightly brownish. This seemed to indicate some chemical change by which acid had been formed which dissolved the CaCO_3 , and also a slight decomposition of the dextrose. Later analyses indicated that a loss of nitrogen in some form may have accompanied this change. In the other solutions no such change took place, indeed the cloudiness increased rather than decreased, due no doubt to loss of carbon dioxide during the boiling. This showed that the change above mentioned was due to the high concentration of the ammonium nitrate.

TABLE X
CULTURES IN VARYING QUANTITIES OF AMMONIUM NITRATE AS INDICATED

No.	Mol. wt. concentration	Amount in 1000 cc. gm.	Amount in each culture (50 cc.) mg.	Calculated nitrogen mg.
1	M/10.....	8.0	400.0	140.19
2	M/50.....	1.6	80.0	28.04
3	M/250.....	0.32	16.0	5.61
4	M/1250.....	0.064	3.2	1.12
5	M/6250.....	0.0128	0.64	0.22

Two fungi were used for these cultures: *Fusarium* and *Myceliophthora*. Analyses were made of the latter only. The cultures were allowed to develop from April 17 to June 5, or 48 days. Vigorous and characteristic growth took place in all the cultures of both fungi.

The *Fusarium* produced the characteristic pink coloration of the medium, the degree of color depending on the amount of growth. At first the M/250 showed the best growth, but later this was surpassed by the M/50, and finally the best growth was in the M/10, although M/10 and M/50 showed but little difference. As previously stated, no analyses were made of this form.

The growth of *Myceliophthora* differed in the fact that the best growth both at the first and at the end was in the M/250 solution. Also the mycelium, at least in the higher concentrations, developed a small amount of purple color on the surface of the mat, while the

liquid itself was made yellowish brown. In the three higher concentrations a thick botryoidal mat was developed.

At the end of the growth period, all of the cultures of *Myceliophthora* were submitted to analysis to determine the amount of nitrogen in the mycelium and in the filtrate. The M/50 culture solution was analyzed for nitrogen content as a check on the purity of the ammonium nitrate. The medium without ammonium nitrate was also analyzed. The mycelium in each case was analyzed by the nitrate-free method, while the nitrate method was used for all the filtrates. The results are all incorporated in table XI.

TABLE XI
DEVELOPMENT PERIOD 48 DAYS

No.	Concentration	Dry wt. of mycelium mg. <i>a</i>	N in the mycelium mg. <i>b</i>	N in the filtrate less medium (12) mg. <i>c</i>	Total N (<i>b</i> + <i>c</i>) mg. <i>d</i>	N supplied as HN_3NO_3 mg. <i>e</i>	N gain mg. <i>f</i>
First series							
1	M/10.....	98.0	5.41	130.85	136.26	140.19	-3.94
2	M/50.....	148.1	7.51	20.08	27.59	28.04	-0.45
3	M/250.....	242.6	4.91	0.70	5.62	5.61	0.01
4	M/1250.....	76.5	1.40	-0.14	1.26	1.12	0.14
5	M/6250.....	26.6	0.70	-0.21	0.49	0.22	0.27
Duplicate series							
6	M/10.....	98.6	5.19	130.71	135.90	140.19	-4.29
7	M/50.....	166.6	7.58	19.66	27.24	28.04	-0.80
8	M/250.....	257.2	5.19	0.42	5.62	5.61	0.01
9	M/1250.....	76.4	1.26	0.00	1.26	1.12	0.14
10	M/6250.....	25.4	0.42	0.00	0.42	0.22	0.20
11	M/50 control, not inoculated, 27.80 mg. N.						
12	Medium without NH_4NO_3 (50 cc.), 0.70 mg. N.						

It is difficult to find any evidence whatever of nitrogen-fixation in these results, even though the combined nitrogen enabled the fungus to begin a vigorous growth. The nearest suggestion of evidence appears in analyses 4 and 5, where the mycelium seems to have gained a slight amount of nitrogen. In the same analyses the filtrates show a slight loss. Whether there is significance in this or whether it is just a coincidence in the errors of two analyses seems hard to say, since the amounts are so small as to give one little confidence in their importance. Furthermore, the duplicate

analyses show still smaller gains. In any case, these results could only show that the fungi under these conditions had been able to use a very small amount of the combined nitrogen, which the analyses of controls has shown to be present in the dextrose. All preceding evidence of this investigation has been against this idea, and we are strongly inclined to hold to the idea that these results are due to slight variations in the accuracy of the method of analysis.

Another interesting and perhaps important fact may be seen from the last table. The amount of nitrogen present in the mycelium is not proportional to the dry weight. This point was suggested earlier in the examination of mycelia grown in nitrogen-free media, where the hyphae looked somewhat shriveled and starved, as if the protoplasm lacked some necessary constituent. It appears here that the mycelium has the power of taking up a higher amount of nitrogen than it really needs for the best growth, judging best growth by the amount of dry weight, for in the two higher concentrations a larger percentage of nitrogen is shown, even though the dry weight is much less than in the M/250 concentration. The amount of nitrogen is also proportionally higher for the M/10 than for M/50. This holds for both series of cultures. If we take the averages of the two series, the per cents of nitrogen in dry weight of mycelium are approximately as follows: M/10, 5.5 per cent; M/50, 5 per cent; M/250, 2 per cent; M/1250, 1.8 per cent; M/6250, 2 per cent. Or, stated more generally, the fungus will assimilate about 2 per cent of its dry weight of nitrogen when this is supplied in such quantities that the fungus can use all that is present, but when the nitrogen is in excess of what the fungus can use, then a larger percentage is assimilated, running as high as 5.5 per cent. The evidence seems too scanty to make certain whether such a generalization would hold in all cases. Why this larger percentage of nitrogen is present in the solutions of higher concentration does not seem apparent. It may be that the presence of nitrogen compounds in excess causes the fungus to use these instead of carbon compounds, which are used in greater quantity when the nitrogen supply is limited. This investigation can only raise this question, and suggest its value as a subject worthy of further physiological investigation.

The loss of total nitrogen in the M/10 and M/50 cultures has been referred to. This may have been due, as already suggested, to some chemical action which gave off some compound of nitrogen during the sterilization. The dissolving of the calcium carbonate and the slight change in the color of the culture solutions suggested this, but no positive evidence of such nitrogen loss was obtained. Another possible explanation might be that nitrogen in some form was given off as a waste product during the metabolism of the growing fungus. However, no evidence of such a hypothesis was observed. This might raise another interesting question for further investigation.

DISCUSSION OF RESULTS IN RELATION TO NITROGEN-FIXATION

As a result of this series of investigations, it may be said that we find no evidence whatever which seems to justify a belief in the power of any of the forms investigated to fix free nitrogen under the conditions used.

The question of why such conflicting results regarding nitrogen-fixation by fungi have been reported by different investigators still remains. This investigation was not carried on to settle this question, but rather because it was hoped that by investigating a large number of forms, and especially those in the soil, some fungi might be found which certainly had the nitrogen-fixing power. There seems no reason a priori why fungi, as well as bacteria, should not have such power, and certainly, reasoning from analogy, there seems much reason to expect it. However, we have been wholly disappointed in this expectation as a result of this series of investigations. Furthermore, while it was not designed to work over forms previously studied, nevertheless, negative results are given here for one form, *Hormodendron cladosporioides*, for which positive results have been reported by others. Both FROELICH (11) and PURIEWITSCH (16) found appreciable nitrogen-fixation for this fungus on both nitrogen-free and nitrogen-poor media. We have been entirely unable to confirm these results.

It seems difficult to explain the widely conflicting results. It may be easy, perhaps, to discard entirely such results as those of LATHAM (20), in which, as pointed out by PENNINGTON (25),

6 similar 50 cc. cultures, having about the same quantities of combined nitrogen supplied to them, are reported to fix quantities of nitrogen all the way from 44.9 to 193.6 mg. This discrepancy is even more striking if we use the figures in column 10 of her "table I," which represent total amounts of nitrogen fixed. Here the results for the same 6 cultures as those above referred to vary between 33.3 and 205.1 mg. LATHAM's explanation that this is due to passing beyond the optimum or critical point in the supply of combined nitrogen in the culture hardly seems satisfactory, especially with the scant evidence at hand on this point. Furthermore, the great excess in the nitrogen gain over that reported for fungi by any other investigator, great even when compared with bacteria, would seem to require creditable confirmation before being finally accepted.

It might also be possible to reject some of the earlier work when methods of sterilization, inoculation, and pure cultures were so poorly perfected. However, the same cannot be said of the painstaking work of FROELICH (11), TERNETZ (19), and others, where accurate methods are reported and analytical data are fully given. It must be admitted, at the same time, that the weight of negative evidence is increasing, and it seems likely that few if any fungi will be found to have nitrogen-fixing power, unless, perhaps, it may be in the case of the mycorrhiza forms, regarding which more evidence is much needed.

This investigation does not seem to add weight to the explanations suggested by PENNINGTON of the conflicting results on this question, namely, that difficulties with the Kjeldahl method of analysis have led to variations in reports, and that differences in the strains of fungi used, suggested by THOM's (33) work, might have led to different results. As seen from previous data of this paper, the method of analysis seems capable of giving a limit of error which is far less than the differences in the results of different investigators. Such accuracy, however, could not be depended upon except in the hands of a skilled chemist, or at least one who had acquired considerable practice with the method. As to the second explanation, a good deal more work needs to be added to that of THOM along the line of modification through different culture

conditions. While this question was only incidental in this investigation, the experience with these fungi under quite a variety of culture conditions makes it hard to think that this can be a satisfactory explanation. Variations did, indeed, occur to some extent, but in all cases each form retained its distinct species characteristics, and showed no signs of nitrogen-fixation under any of the conditions.

If nitrogen-fixation is finally established for any fungous forms, it seems probable that the character of the nutrient material supplied may be found to be an important factor. It is not felt that this investigation supplies sufficient data along this line to permit final conclusions. There is also the possibility, as already suggested, that the nitrogen-fixing power may be found to be confined wholly to the mycorrhiza forms.

IV. Summary of results

This series of investigations would seem to justify the following generalization of results:

1. Many species of fungi live habitually in the soil, carrying out their life history there, either in whole or in part. A considerable number of these have been found, so far, only in the soil.

2. These fungi are, at least to quite an extent, uniform in different soils, and, unlike the bacteria, appear to be rather uniformly distributed at different depths, at least as low as 14 cm.

3. Tillage and manuring seem to produce little change in the number or kind of these fungi. This conclusion is not regarded as final.

4. These fungi may be cultivated and isolated as pure cultures, without interference from bacterial growth, by the use of 20-30 per cent of gelatin in the culture medium.

5. None of the forms studied, including at least 14 species, shows any power of assimilating free nitrogen when grown in nitrogen-free media under the conditions of these investigations. *Myceliophthora* and probably *Fusarium* show no such power even in nitrogen-containing media.

6. *Myceliophthora* when growing in nitrogen-containing solutions assimilates different proportions of nitrogen in different con-

centrations of the nitrogen compound. The nitrogen assimilated is approximately 2 per cent of the dry weight of the mycelium in all concentrations where the fungus is able to use all the nitrogen, in this case up to and including M/250. In higher concentration, where the nitrogen is in excess of what the fungus can use, the amount of nitrogen assimilated increases up to 5.5 per cent in the case of the M/10 concentration. Also, the optimum growth as indicated by dry weight occurred where the fungus could use all the nitrogen, in which case the amount of nitrogen assimilated was 2 per cent of the dry weight.

7. The amount of combined nitrogen taken up from the air, by cultures standing exposed, does not seem to be sufficient to make appreciable difference in their nitrogen content, either in nitrogen-free or in nitrogen-containing media.

8. These fungi do not seem to be able to use nitrogen in all its forms, since analysis failed to show that they could use that present in the dextrose of the culture medium.

9. The Kjeldahl method of analysis is capable of a degree of accuracy which will reduce the limit of error very near to 0.1 mg. for each determination in analyses involving very small quantities of nitrogen. In analyses involving larger quantities of nitrogen, the error may be reduced to 0.3 of 1 per cent.

10. A very perceptible growth of mycelium is possible in practically nitrogen-free media, but in such cases the nitrogen content is found by analysis to fall within the limit of error of the method. Furthermore, the mycelium shows a starved, shriveled condition, as if deficient in some necessary element. In these cases, mycelia having a dry weight of 3-6 mg. gave amounts of nitrogen within the limit of error. Conversely, this may be something of a qualitative index of nitrogen-fixation, for, when the dry weight of mycelium is not more than 6-8 mg., there is little or no probability of nitrogen-fixation.

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